GENETIC AND ECOLOGICAL CONSEQUENCES OF A SHIFTED PHENOLOGY IN A FOREST DEFOLIATOR

SUSANA CRISTINA MACHADO ROCHA

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TESE ELABORADA PARA OBTENÇÃO DO GRAU DE DOUTOR EM ENGENHARIA FLORESTAL E DOS RECURSOS NATURAIS

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Abstract

Thaumetopoea pityocampa is a major pine defoliatior over the Mediterranean Basin. An atypical population with a shifted phenology, ongoing an allochronic differentiation process, was recently discovered in Mata Nacional Leiria, Portugal. The shifted population reproduces in spring achieving its larval development during summer (SP), whereas the sympatric typical population reproduces in summer and has winter larval development (WP). They are reproductively isolated through time, although sharing the same space and host species. General objectives of this work were to characterize the current and potential distribution range of this population, its spatial/temporal genetic and demographic patterns, and ecological adaptations.

Field monitoring of presence/absence of SP nests throughout Leiria showed that it is expanding to North and South. Current distribution is restricted to coastal areas, along ca. 120 x 20 km area. Distribution models predict that its distribution will be restricted to coastal areas, due to unsuitable climate inland, being the maximal temperatures of the summer months the main constraint.

A phylogeographic study confirmed high SP differentiation from other Portuguese populations. A spatio-temporal sampling along two geographic transects in Leiria zone revealed high stability in time of the genetic composition and structure. A striking demographic result showed that SP tends to apparently displace the WP from the coastal zones suggesting a possible competition phenomenon. Gene flow between the two populations was shown to remain low, even if some SP males actually emerge late.

Ecological differentiation was assessed at egg and larval stages. The effect of a wide range of high temperatures on egg survival and development showed higher performances of the SP. Furthermore, SP larvae showed a better ability to cope with host plant water stress, evidencing adaptation to summer feeding activity.

This study highlights intra-specific variations on the response of this insect to environmental constraints evidencing ecological adaptation following allochronic differentiation.

Keywords: allochrony, climate change, genetics, species distribution models, *Thaumetopoea pityocampa*

Resumo

Título: Consequências genéticas e ecológicas do desvio na fenologia de um insecto desfolhador

O género *Thaumetopoea* engloba cerca de 10 espécies distribuídas geograficamente por vários países Europeus e da Bacia do Mediterrâneo, diferindo entre si não só em relação às espécies hospedeiras bem como a particularidades do seu ciclo de vida. De entre as várias espécies, a processionária do pinheiro, *Thaumetopoea pityocampa* (Den. & Schiff.) (Lepidoptera, Notodontidae), é um dos mais importantes desfolhadores de coníferas, em particular de pinheiros, encontrando-se em países da bacia do mediterrâneo. Da sua atividade resultam danos nas árvores com consequente redução do seu crescimento radial, tornando-as mais susceptíveis a pragas secundárias. Este insecto constitui também um problema de saúde pública uma vez que as larvas desenvolvem pêlos urticantes responsáveis por provocar alergias em pessoas e animais.

A processionária do pinheiro tem um ciclo de vida anual, no qual os adultos emergem nos meses de Verão reproduzindo-se de imediato e efectuando a postura dos ovos que ao fim de cerca de um mês dão origem a larvas recém-eclodidas. O desenvolvimento larvar decorre de Outubro a Fevereiro-Março, desenrolando-se por 5 instares em que as larvas vivem em ninhos de forma gregária. No final do Inverno deslocam-se em direcção ao solo onde se enterram permanecendo em diapausa no estado de pupa durante 3 a 5 meses.

Em Agosto de 1997, foi observado na Mata Nacional de Leiria (MNL) um ataque muito severo de processionária do pinheiro (M.R. Paiva, observação pessoal). A descoberta foi intrigante devido à presença de larvas em 5º instar, numa altura do ano que não correspondia ao ciclo habitual da espécie. Verificou-se que esta população tinha um ciclo de vida desfasado do habitual, com os adultos a surgirem na Primavera, o desenvolvimento larvar a ocorrer no Verão e as larvas a enterrarem-se no Outono para pupar. Por ter desenvolvimento larvar no Verão esta população passou a designar-se por ”população de verão” (SP) em contraste com a população típica, com desenvolvimento larvar no Inverno e por essa razão designada por ”população de inverno” (WP). Desde então tem-se verificado que SP se tem vindo a expandir tanto para Norte como para Sul da área específica da MNL. Estudos anteriores realizados com marcadores genéticos (COI e ITS) permitiram verificar que esta população pertence à espécie *T. pityocampa* e pertence ao mesmo clade ibérico das restantes populações portuguesas Estudos com

microssatélites revelaram, todavia, uma forte diferenciação entre SP e a população simpátrica de Leiria WP assim como de outras populações WP. Os dados sugerem ainda que SP se terá estabelecido a partir de um número reduzido de indivíduos WP que terão tido um desvio na sua fenologia, nomeadamente ao nível reprodutivo. Verificou-se que as duas populações simpátricas se encontravam isoladas do ponto de vista reprodutivo, utilizando no entanto o mesmo habitat e alimentando-se das mesmas espécies hospedeiras, o que faz desta população um caso raro de especiação alocrónica em curso. Estudos de campo utilizando armadilhas para captura de machos, permitiram verificar que o intervalo entre o período reprodutivo das duas populações é muito curto (cerca de 2 semanas) levantando a questão da possibilidade de ocorrência de hibridação devido a indivíduos que emergem mais cedo ou mais tarde possibilitando a ocorrência de fluxo genético. Estudos efectuados em laboratório com o objectivo de estudar os padrões fenológicos das duas populações e efectuar cruzamentos híbridos, permitiram concluir sobre a elevada hereditariedade da fenologia, tendo os indivíduos híbridos características intermédias. Amostragens de campo, permitiram identificar alguns (poucos) indivíduos híbridos, principalmente nos limites a Norte e a Sul da sua distribuição, isto é, em áreas recentemente colonizadas. Uma das consequências da alteração da fenologia da população SP é o facto de se encontrar sujeita a diferentes pressões selectivas quando comparada com outras populações, implicando por isso adaptações evolutivas reforçando a diferenciação desta população. Estudos anteriores revelaram a ocorrência de diferenciação ecológica das duas populações ao nível dos estádios de ovo e larva. Nomeadamente, observou-se divergência ecológica na fecundidade, tamanho e cobertura dos ovos, bem como no parasitismo óofago, da população SP. Larvas dos instares mais jovens, L1 e L2 da população de Verão, demonstraram uma maior tolerância a temperaturas elevadas quando comparadas com larvas dos mesmos instares das populações de Inverno. Todos estes resultados indicam a presença de um processo de especiação alocrónica incipiente com diferenciação ecológica em curso.

No presente estudo visou-se esclarecer questões em aberto acerca do sucesso da população de Verão, SP. Em particular pretendeu-se caracterizar a sua actual e potencial distribuição futura, os seus padrões genéticos e demográficos tanto no tempo como no espaço, bem como alguns dos aspetos de adaptação ecológica na fase de ovo e de larva.

Como primeiro objetivo visou-se estudar os padrões de dispersão da população SP, bem como a sua probabilidade de ocorrência noutras regiões nas actuais e futuras condições

climáticas. Para tal fez-se uma monitorização intensiva, da presença/ausência desta população, com base na observação de ninhos de 5º instar com larvas vivas num total de 131 pontos de amostragem numa extensão com cerca de 150 Km, estendendo-se para Sul, Norte e Oeste da região da MNL. Os resultados mostraram que a população se encontra a expandir tanto para Norte como para Sul da sua área de distribuição inicial, na MNL. A sua distribuição actual está restrita às áreas costeiras numa faixa com cerca de 120 x 20 Km. A sua taxa de expansão quer para Sul quer para Norte a partir da área onde inicialmente foi descoberta, foi de 2.51 e 1.66 $km.ano^{-1}$, respectivamente, durante o período de 1997-2014. Foram utilizados modelos de dispersão de espécies (GLM, CART, BRT, MAXENT) que predizem que a sua distribuição futura ficará restrita às áreas costeiras, devido a uma maior instabilidade das condições climáticas das áreas com localização mais interior. Os modelos prevêem que as temperaturas máximas dos meses de Verão, cada vez mais elevadas devido às alterações climáticas, serão o principal obstáculo à expansão desta população para outras áreas.

A realização de um estudo filogeográfico permitiu confirmar a elevada diferenciação da população SP quando comparada com outras população WP localizadas de Norte a Sul do País, incluindo a população WP de Leiria. Uma amostragem espácio-temporal efectuada com recurso a dois transectos geográficos com cerca de 100 Km cada e distanciados cerca de 20 Km, instalados na região de Leiria, um em zona costeira com 9 pontos de amostragem e outro paralelo em zona mais interior com 7 pontos de amostragem, ao longo de dois anos (2012 e 2014), revelou uma elevada estabilidade temporal da estrutura e composição genética desta população. Um resultado demográfico relevante foi o facto de se ter observado que nos locais onde a população SP se encontra em elevada densidade, a população WP é quase residual, sugerindo uma aparente deslocação desta população das zonas costeiras, configurando um possível fenómeno de competição. Foi demonstrado que o fluxo genético entre as duas populações permaneceu baixo, mesmo com alguns machos SP a emergir mais tarde.

A diferenciação ecológica foi avaliada tanto ao nível dos ovos como das larvas. O efeito de uma ampla variedade de elevadas temperaturas (36ºC a 48ºC) testadas no desenvolvimento e sobrevivência dos ovos em três populações com fenologias distintas (duas populações Portuguesas, SP e WP, e uma população Tunisina), demonstrou uma melhor performance da população SP. Esta população evidenciou maior sobrevivência dos ovos em todas as temperaturas testadas (incluindo a temperatura controlo a 25ºC), comparativamente às populações WP. Permitiu também definir o limite térmico

máximo para os ovos desta espécie, como sendo de 42ºC, constituindo uma mais-valia para a predição da expansão potencial deste insecto no cenário das alterações climáticas. Na fase larvar analisou-se a resposta desta população ao stress hídrico das plantas hospedeiras, dado que o seu desenvolvimento larvar ocorre num período estival de secura. Larvas jovens (L1 e L2) de ambas as populações, SP e WP de Leiria, foram alimentadas com plantas hospedeiras (*P. pinaster*) sujeitas a stress hídrico e comparadas com um controlo. Os resultados demonstraram um efeito negativo do stress hídrico da planta hospedeira na performance das larvas de ambas as populações, avaliada ao nível da sobrevivência, taxa de consumo e tempo de desenvolvimento larvar. No entanto, a população SP demonstrou uma maior capacidade para lidar com o stress hídrico do hospedeiro, não evidenciando diferenças significativas na sobrevivência e desenvolvimento das larvas entre os dois tratamentos. No geral, a população SP mostrou ainda menor mortalidade que a população WP. Estes resultados evidenciam a ocorrência de variações intra-especificas na resposta ao estado da planta hospedeira como resultado de mecanismos de adaptação ecológica.

Do ponto de vista evolutivo, este trabalho ofereceu uma oportunidade para analisar e acompanhar os mecanismos geográficos, genéticos e adaptativos de um processo de especiação alocrónica em curso, raramente documentado na natureza.

Palavras-chave: alocronia, alterações climáticas, genética, modelos de distribuição, *Thaumetopoea pityocampa*

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INTRODUCTION

Introduction

The work developed in the present thesis focuses on a population of the pine processionary moth (PPM), *Thaumetopoea pityocampa* (Den. & Schiff.) (Lepidoptera, Notodontidae) experiencing a process of allochronic differentiation, i.e., which is reproductively isolated from other PPM populations due to a shift in the reproductive period. We will here address questions regarding its geographical distribution and expansion patterns, as well as its genetic structure and specific ecological adaptations. In the present introduction, an overview of the background knowledge is presented on this model species with a special attention to recent advances related to this allochronic population. We will detail the results gained from previous studies regarding genetic, ecological and geographical aspects and present the general objectives of the present work, which aims at unraveling some of the prevailing unknowns.

Thaumetopoea pityocampa

The genus *Thaumetopoea* constitutes a group of important defoliators that cause serious damage both to conifers and broad-leaved trees, depending on the clade they belong to. This genus encompasses ca. 10 species (Simonato et al., 2013), distributed across several European and Mediterranean countries.

Life histories of *Thaumetopoea* species can be distinguished by the biannual or annual type of life cycle and the existence of either embryonic diapause (as *Thaumetopoea processionea* (Linnaeus), *T. bonjeani* (Powell), *T. solitaria* (Freyer) and *T. herculeana* (Rambur)), or pupal diapause (as *T. pityocampa* (Denis & Schiffermüller), *T. wilkinsoni* (Tams) and *T. jordana* (Staudinger)), or both (*T. pinivora* (Treitschke)). Depending on species, larvae feed either in fall and winter (e.g. *T. pityocampa*, *T. wilkinsoni*) or in spring (e.g. *T. pinivora*, *T. processionea*). Within each species, dates of adult emergence and reproduction, and season of larval development show some variability depending on geographical and altitudinal distribution (Douma-Petridou, 1989; Schmidt, 1989; Halperin, 1990).

Among *Thaumetopoea* species, the pine processionary moth (PPM), *T. pityocampa* is one of the main defoliators of conifers. It occurs all over the western part of the Mediterranean Basin, reducing tree growth and consequently forest yield (Kanat et al., 2005; Gatto et al., 2009; Arnaldo et al., 2010; Jacquet et al., 2012). Economic losses can

even be more significant as the weakened trees become highly prone to attacks by secondary pests (Kanat et al., 2005; Arnaldo et al., 2010). This insect species further causes concern for public health due to the urticating hairs of the larvae, which is the most important impact in urban and recreational forest areas (Vega et al., 2004).

Over its whole range, including Portugal, *T. pityocampa* has a typical annual life cycle in which adult emergence occurs in the summer, immediately followed by reproduction and egg-laying. Larvae hatch in about one month and develop through fall and winter, living gregariously and constructing silk nests to cope with the low winter temperatures. Larvae develop through 5 instars during three to eight months. Between January and March, they leave the trees in long processions until finding a suitable place in the ground to bury and pupate. Pupal diapause is an obligate stage, and lasts for three to five months, which allow the adults to emerge at the same period in summer. Small variations in the phenology are linked to climatic characteristics as later adult emergences were observed in warmer climates, and earlier emergences along higher latitudinal and altitudinal ranges (Démolin, 1969; Huchon & Démolin, 1970).

A population exhibiting a shifted phenology in Portugal

In August of 1997 an anomalous population was detected in a forest stand of the Mata Nacional Leiria (MNL), Portugal (Fig. 1A), with a very high density of L5 individuals, reaching a rarely observed population level (Paiva, pers comm.; Pimentel et al. 2004). Trees were highly defoliated, hosting a high density of nests and larvae, which was particularly intriguing considering the date of these observations. Indeed, only eggs or just hatching small larvae are usually observed in the field at that time of the year.

Since its discovery, this SP population has been studied by several teams working in close collaboration, namely ISA-UL, FCT-UNL and INRA. Field observations and male monitoring using pheromone traps were then set up to characterize this population and determine if individuals following the typical life cycle were also occurring. These data showed that this unique population is characterized by adult emergence and oviposition during the spring (May and June). Larvae start hatching around mid-June and develop throughout the summer. Processions and pupation in the soil usually take place as early as mid-September, adults emerge again in May or June the following year (Pimentel, 2004; Pimentel et al., 2006). As larval development occurs during the summer months, this population was called the "summer population" (SP). Interestingly, the data

gathered showed that a fraction of individuals still followed the typical phenology, and constitute the Leiria "winter population" (WP) (Fig. 2).

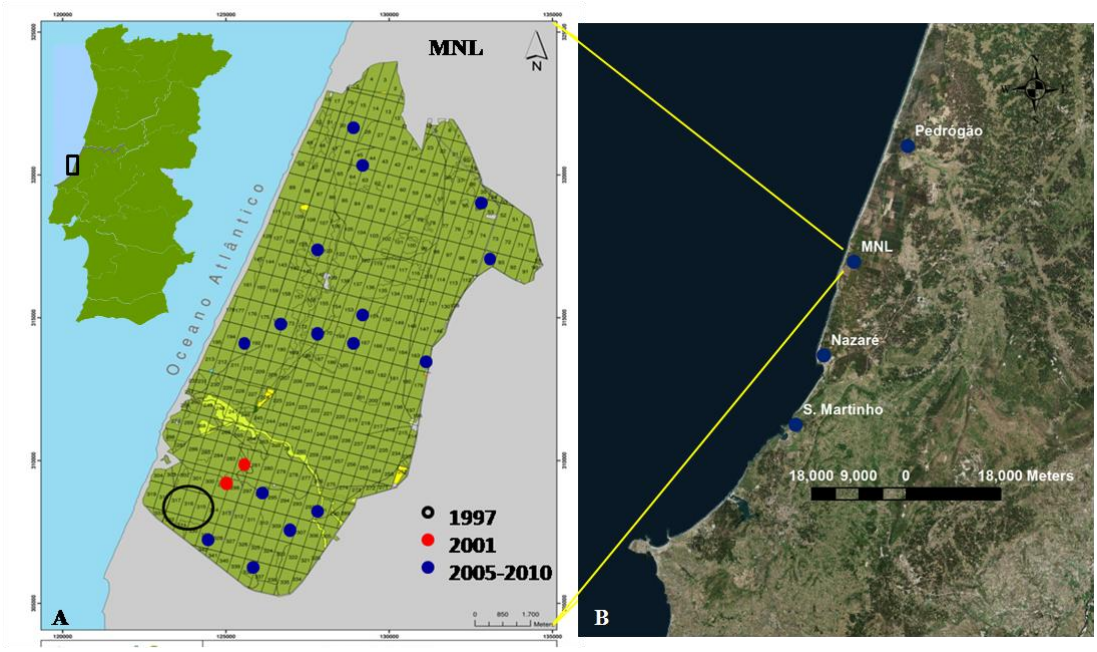

Figure 1 – Recorded presence of SP population. A: Records from 1997 to 2010 within the Mata Nacional Leiria (MNL); black circle highlighting the area where SP was discovered in 1997. B: Whole known range in 2010.

Figure 2 - Comparative phenologies of the two *T. pityocampa* populations in Portugal: WP – Winter Population, SP – Summer Population.

It is worth noting that all other populations in Portugal follow the typical life cycle and are thus Winter Populations. Although WP occurred in relatively low densities in MNL, the co-existence of the two populations was noticeable. The following years, after 1997, SP consistently exhibited high densities in its core area and expanded southward and northward of the Mata Nacional Leiria (MNL) (Pimentel, 2004; Santos, 2004; Pimentel et al., 2006). Although with lower densities, it was found for the first time in 2005 in Nazaré and S. Martinho just South of MNL, and, in 2007 in Pedrógão, North of MNL (Fig. 1A-B), as demonstrated by monitoring male flight activity for several years using funnel traps (Santos et al., 2007; Santos, 2012).

The existence of this population raised many questions concerning its origin and evolutionary history. One working hypothesis was that it came from an introduction from a distant area, possibly followed by local hybridization and introgression. It was thus thought to be either a cryptic species or an introduction of a differentiated sub-clade of *T. pityocampa*, for instance from North Africa.

Genetic differentiation studies

Initial research, based on mitochondrial and nuclear sequences, showed that the SP belongs to the same species as the surrounding WPs, with no sequence differentiation between the two (Santos, 2004; Santos et al., 2007). However, some few microsatellite markers revealed a strong differentiation between SP and WP, with signs of founder event in the SP, suggesting that this population was recently established by a reduced number of WP individuals with early sexual reproduction, becoming thereafter strongly genetically differentiated due to a reproductive isolation through time, i.e. allochronic differentiation (Santos et al., 2007, 2011a). Santos et al. (2011a), monitoring the flight activity of the adults, hypothesized complete reproductive isolation of the two populations. The authors nonetheless considered that gene flow could occur via individuals shifting from one phenology to the other, or due to a small window of overlapping emergence times between the 2 populations. The nuclear markers then available were not appropriate to finely assign individuals to potential hybrid categories.

In a more recent study, Burban et al. (2016) confirmed very low mitochondrial differentiation between the sympatric SP and WP, with most individuals from the Leiria zone sharing the same main haplotype whatever their phenology. This was interpreted as a sign of the very local origin of the SP from the local WP population, but this could

also be due to local recurrent introgression. Using recently developed microsatellite data, the authors showed a strikingly high differentiation between the SP and a few other WP populations sampled from Portugal. Genetic assignations allowed the identification of individuals that phenologically corresponded to WP (i.e., trapped from late July to September) but genetically belonged to the SP cluster. Such remarkable individuals were designated as "LateSP". It was suggested that they could either result from longer pupal diapause or from diapause dysfunction. Importantly, few individuals were identified as hybrids (F1, F2 or back crosses) located mainly in the southern and northern limits of the SP distribution, this is, in recently colonized areas (Burban et al., 2016). All these results suggested that an allochronic speciation process was ongoing in the Leiria region. The demonstrated genetic differentiation between the SP and the WP was indeed recently acknowledged as a very promising example of true allochronic speciation in the review made by Taylor & Friesen (2017). Allochrony can be seen as a magic trait causing or reinforcing reproductive isolation by displacing mating time. It has been so far overlooked, because it can be difficult to detect. The situation in the Leiria region provides an unprecedented opportunity to follow the first step of an allochronic differentiation, which is still under way. To complete the picture, it was important to check if adaptive phenotypic differentiation was also occurring.

Ecological adaptations

One main consequence of the phenologial shift is that individuals from the SP are now exposed to different selection pressures compared to other populations, because the various life stages do not occur under usual environmental conditions. Such new selection pressures could favor the evolution of adaptations, reinforcing the divergence of this population. In particular, summer larval development exposes young larvae to temperatures much higher than those of the typical WPs. An increased tolerance to high temperatures was actually demonstrated for the early L1 and L2 larval stages of the SP compared to other WP populations (Santos et al., 2011b). This suggests that adaptation to high temperatures could and did occur since the SP foundation (Santos et al., 2011b). Moreover, Santos et al. (2013) also showed that the SP had peculiar characteristics regarding reproduction, such as a lower fecundity, larger eggs and a different egg cover that could be an adaptation to the lower spring temperatures. According to Santos (2012), results evidence the occurrence of rapid phenotypic differentiation following

exposure to different ecological constraints. Other types of pressures, as from natural enemies, may also condition future evolutionary steps. Santos et al. (2013) found that SP eggs are less exposed to parasitism compared to those of WP, due to a desynchronized life cycle of the most common egg parasitoids.

The identification of the role of different ecological factors in the survival and performance of the SP is a matter of great interest that might contribute to further understand differentiation of the two populations.

Objectives

The purpose of this work was to enhance our knowledge about the success of the SP population, and to characterize its capacities to colonize and expand to other regions. As the SP larvae feed in summer, we also tested whether they could better cope with plant water stress than the WP individuals. We hypothesize that ecological adaptations to summer larval development could have occurred and favor the population with shifted phenology. This would bring useful knowledge to anticipate the possible fate of the SP and question its interactions with the sympatric WP. From an evolutionary point of view, this will give us the opportunity to analyze the mechanisms of an ongoing allochronic speciation, which has been rarely documented in nature. Understanding some underlying mechanisms will also provide tools to forecast the possible occurrence of similar phenomena in other regions. Specific objectives are to:

i) Assess the dispersal patterns of SP as well as its probability of occurrence in other regions under current and future climatic conditions (Chapter 1);
ii) Analyze the genetic structure of the sympatric SP and WP using a systematic sampling design, follow their evolution in space and time and characterize potential LateSP or introgressed individuals (Chapter 2);
iii) Explore and characterize ecological adaptations between the two sympatric populations, regarding embryonic development (Chapter 3)
iv) Determine how larvae of the two sympatric populations cope with host plant stress, and whether phenotypic differences can be identified regarding this constraint (Chapter 4).

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CHAPTER 1

Climate constrains range expansion of an allochronic population of the pine processionary moth

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Climate constrains range expansion of an allochronic population of the pine processionary moth

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Abstract

Allochronic speciation occurs when sympatric populations sharing similar feeding regimes diverge because they mate at different times. Such speciation mode is fascinating because it permits the study of subsequent adaptations and/or geographic range shifts undergone by the populations that face new ecological constraints. Moreover, exploring whether intraspecific differentiation is accompanied by niche divergence is crucial for planning efficient biodiversity management and invasive species control. Here, we address the topic of potential climatic niche divergence between two sympatric populations of the pine processionary moth *Thaumetopoea pityocampa* (PPM) that diverged under allochrony. The larval development of a PPM population recently discovered in coastal regions of Portugal, referred to as the summer population (SP), occurs in the spring–summer instead of the autumn–winter as for all other populations of this species. We carried out intensive field sampling in the area where both populations are sympatric and calibrated spread (MigClim approach) and species distribution models (ensemble forecasting approach) to depict the response of SP to environment and predict its potential range under current and future climatic conditions. Since its discovery in 1997, the distribution of SP has been expanding along the Western coasts of Portugal. Still, its establishment in inland regions failed, even though suitable hosts occur. Our models identify maximal temperatures as the main constraint explaining SP absence from inland regions. The coastal area where winter populations and SP co-occur displays unique climatic

conditions (moderate maximal and minimal temperatures), which enable the coexistence of these populations with totally different phenologies. The models predict a future decrease of SP range due to climate warming. We show here that a climatic niche shift occurred following allochronic divergence. This study highlights how climate differentially affects allochronic populations and how considering intraspecific diversity is crucial when predicting species responses to climate change.

Keywords: allochronic speciation, intraspecific diversity, MigClim, niche shift, phenology change, *Thaumetopoea pityocampa*

Introduction

There is increasing evidence that intraspecific diversity should be accounted for when the potential distribution of species is predicted for purposes of conservation or pest management. Disregarding the potentially different responses of genetically divergent populations to environmental conditions may lead to inefficient biodiversity management plans (Benito Garzon et al., 2011; D'Amen et al., 2013) or invasion risk assessments (Peterson & Holt, 2003; Rey et al., 2012; Godefroid et al., 2015, 2016). Allochronic speciation is an increasingly documented important driver of intraspecific differentiation in several groups of organisms (Miyatake et al., 2002; Abbot & Withgott, 2004; Yamamoto & Sota, 2009). Allochronic speciation arises when sympatric populations with similar feeding regimes and similar habitat requirements diverge because they mate at distinctly different times (Alexander & Bigelow, 1960). Populations that have experienced a drastic shift in reproduction time are expected to have overcome new ecological barriers through adaptations (Santos et al., 2011b) and/or subsequent geographic range shifts. For that reason, exploring niche dynamics during allochronic speciation is an attractive field for evolutionary ecologists and provides an ideal context to understand the response of organisms to environmental changes and forecast the impact of ongoing global warming upon biodiversity. The pine processionary moth (PPM), *Thaumetopoea pityocampa* (Denis & Schiffermüller) (Lepidoptera: Notodontidae), is a pine-feeding insect that occurs throughout southern Europe and northern Africa, including the Atlantic coast (Kerdelhué et al., 2009). Adults typically emerge and reproduce during summer, and larvae develop during fall

and winter (Démolin, 1969). An obligate nymphal diapauses occurs until adult emergence takes place in the following summer (Démolin, 1969). The PPM exhibits this phenology over its whole range, and such typical populations are referred as to the 'winter populations', hereafter WPs (Kerdelhué et al., 2009). In 1997, an atypical population exhibiting divergent phenology was discovered in Portugal in Mata Nacional Leiria (National Forest Park of Leiria), hereafter MNL, where it was sympatric with WP (Pimentel et al., 2006) and fed on needles of *Pinus pinaster* (Aiton). Larvae of this newly discovered population develop during the summer while adults mate in the spring. This unique population is referred to as the 'summer population', hereafter SP, in opposition to the WP. In August 1997, the SP was found in an outbreak condition, yet its distribution was restricted to a few squared km inside MNL (M.R. Paiva, pers. obs.). Since the discovery of the SP, its geographical range has slowly expanded along the Western coast (Battisti et al., 2015). Today the range of the SP is restricted to a coastal section of central Portugal, while WPs occur wherever pines are present, that is over most of Portugal. No other population of PPM with such abruptly shifted life cycle has been observed in other regions of either Europe or North Africa. Both the SP and the sympatric WP exhibit low phylogenetic differentiation based on nuclear internal transcribed spacer 1 (ITS-1) and mitochondrial cytochrome oxidase 1 (CO1) (Santos et al., 2007; Burban et al., 2016), which suggests a recent, local origin of the SP. However, the two sympatric populations are highly differentiated based on population genetics data (microsatellite markers), suggesting a founder effect and limited gene flow (Santos et al., 2007, 2011a; Burban et al., 2016). Based on these results, it was hypothesized that mutation(s) in a phenology-related gene(s) caused the relatively recent local foundation and emergence of SP, which is further consistent with high heritability of the shifted phenology (Branco et al., 2016). Allochrony then abruptly disrupted gene flow between the sympatric populations that subsequently diverged in several adaptive and morphological traits (Santos et al., 2011b, 2013). The currently observed system is thus likely to reflect the incipient stage of an ongoing allochronic speciation (Santos et al., 2007, 2011a). The PPM is, consequently, an ideal organism to study the response of closely related populations facing new environmental selective pressures. However, the response of SP to the environment as well as the ecological factors allowing WP and SP to co-occur locally in Portugal are still poorly understood (but see Santos et al., 2011b, 2013). Understanding the ecological aspects of allochronic divergence requires accurate depictions of both the geographic range and the ecological niche of the SP, which are

currently lacking. Additionally to being an ideal biological model for research purposes, the PPM is an economically important defoliator species that causes large losses to pine forests (Hódar et al., 2002) and severe allergic reactions in humans due to the urticating setae released by the larvae (Vega et al., 2004). In the SP, the urticating life stages occur at the end of the summer, coinciding with the main touristic season, by contrast with the typical WP larvae that are present during the colder months. The SP deserves higher attention from biosecurity agencies as this population is currently expanding in Portugal and, consequently, may constitute a serious threat for forestry, public health and tourism economy. While climate tolerances of WP are well depicted and predictions of its future potential distribution are available (Robinet et al., 2007; Battisti et al., 2015), climatic niche requirements of the SP are still poorly understood impeding an accurate prediction of its future distribution. Management of the SP might be impaired by these knowledge gaps regarding its present and future distribution across Portugal and the Mediterranean basin. Predictions of SP future spread are thus required for the development of management programs of this recently discovered population. In this study, we explore the potential intraspecific climatic niche divergence between the allochronic WP and SP, and made predictions about the evolution of the SP distribution range over time. For that purpose, we (1) performed an intensive field sampling to document the current distribution of SP in Portugal; (2) ran multivariate analyses and calibrated species distribution models (SDMs) to depict the response of SP to climate and identify the underlying environmental constraints acting on the geographic range of this population; (3) fitted spread models (MigClim approach; Engler & Guisan, 2009) to predict the potential spread of SP with regard to climate and landscape constraints, as well as dispersal abilities; (4) used SDMs to forecast the potential range of SP under future climate conditions.

Material and methods

Distribution data

The range of SP has been closely monitored since the date of its first detection in Portugal by field surveys and pheromone traps (Pimentel et al., 2006; Santos et al., 2007, 2011a; Battisti et al., 2015; Burban et al., 2016). Based on this information, we carried out an intensive field sampling at the end of the summer 2014, to depict as

accurately as possible the limits of the SP distribution. A total of 131 sampling stations separated by short distances (ranging from circa 0.5 to 4 km) were selected according to the presence of pine stands (Appendix S1, Supporting Information). As our sampling was primarily focused on the borders of the SP range, distances separating neighbour stations within the core of SP distribution were occasionally superior to this range of values. Sampling effort in each station consisted in a thorough inspection of pine stands within a ca. 500-m radius circle for assessment of SP presence/absence. In addition, we performed road sampling of SP nests, a technique that is routinely used to assess the expansion of the WP in northern France (Rousselet et al., 2013). This approach consisted in driving along the main roads between sampling stations, making frequent stops to assess with naked eyes or using binoculars the presence/absence of SP nests in pine stands boarding the roads.

Fifty-seven occurrences were obtained from this campaign and from the different field surveys performed during the period 1997–2014 (Fig. 1 and Apendix S1). Our dataset was restricted to occurrences of nests with living larvae because this kind of record constitutes a good indicator that a PPM population can truly get established in a given locality (contrary to trap records that may encompass individuals that passively or actively dispersed into unsuitable regions). Transferability of correlative bioclimatic models highly increases when models are calibrated with 'true' absences (climatically unsuitable areas for the establishment of an organism) rather than with 'false' absences (climatically suitable localities where an organism is not present because of dispersal constraints or biotic interactions; Elith et al., 2010). We consequently constructed two absence datasets to perform our analyses. A first absence dataset ('ABS1') was assumed to include only true absences and used to calibrate SDMs. We rasterized the minimum convex polygon encompassing presence records with a resolution of 30 arc seconds ('mcp-raster'). The ABS1 dataset comprised the absences located outside this mcp-raster and not situated around the northern and southern fronts of expansion of SP (Fig. 1). Given the recent emergence of SP in Portugal, the absence of this population in regions situated at higher and lower latitudes in this country might, indeed, be explained by dispersal constraints rather than climatic unsuitability of these regions. However, we included in ABS1 most of the absences recorded eastward of the current distribution of SP dataset, as these points were located in regions where the host *P. pinaster* occurs, at distances from the area where SP was first discovered in MNL that were inferior to the maximum distances of spread reached between 1997 and 2014. This fact indicates that

SP spread to inland regions can neither be attributed to its dispersal ability nor to host-related constraints. In fact, host-pine plantations of *P. pinaster* extend almost continuously over MNL and surrounding areas (M. Godefroid, pers. obs.) (Fig. 1). The second absence dataset ('ABS2') comprised the absences of the ABS1 dataset associated with the absences located at the northern and southern expansion fronts of the SP (Fig. 1). The ABS2 dataset was exclusively used to evaluate the predictive power of spread models.

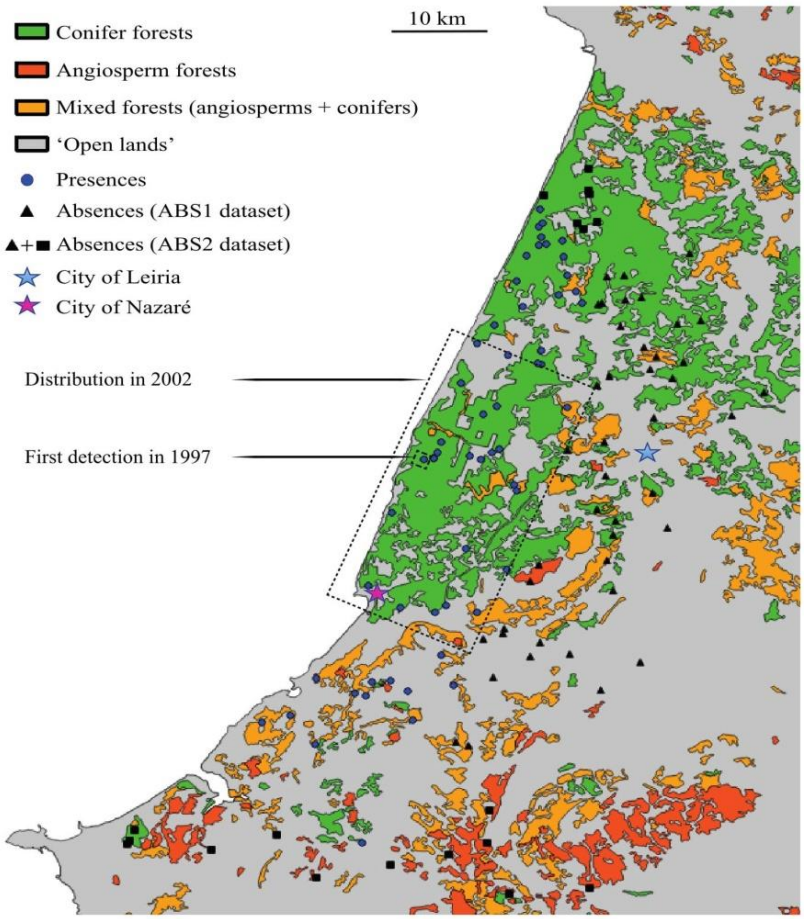

Figure 1 - The presences and absences of the summer population (SP) of *Thaumetopoea pityocampa* used in species distribution and spread modelling. All absence data were obtained during field sampling performed in 2014. Two absence datasets were constructed. The 'ABS1' dataset comprised the absences located outside the minimum convex polygon encompassing presence records and not situated around the northern and southern fronts of expansion of SP. The second absence dataset ('ABS2') comprised the absences of the ABS1 dataset associated with the absences located at the northern and southern expansion fronts of SP.

Landscape and bioclimatic data

We used current and future bioclimatic variables from WORLDCLIM version 1.4 at a resolution of 30 arc seconds (Hijmans et al., 2005). We selected estimations of future climate used in the Fifth Assessment Report (AR5) of the Intergovernmental Panel on Climate Change (IPCC). To enhance the transferability of our bioclimatic models, we calibrated SDMs with a restricted climatic dataset encompassing four ecologically relevant variables according to the biology of the SP. We selected the mean temperature of the warmest month ('bio5') since heat stress is thought to constraint the range of SP (Santos et al., 2011b). Average minimal temperatures recorded in May ('bio20') were considered since the reproduction of SP occurs during spring. To convey a possible moisture stress for pupae, we integrated the mean annual precipitation of the coldest quarter ('bio19') in our climatic dataset. Finally, mean temperature of the coldest month ('bio6') was selected since cold strongly constrains the geographic range of WPs, to test whether this variable could also affect the SP (Battisti et al., 2005). The climatic data from WORLDCLIM (annual trends between 1950 and 2000) do not perfectly reflect the conditions experienced by the SP over the period 1997–2014. We constructed a set of new climatic rasters ('NC bioclimatic rasters') by averaging the 1950–2000 climatic layers with layers corresponding to predictions of future climate by 2020, under the A2 scenario used in the Fourth Assessment Report of the IPPC. These predictions were obtained from the global climate model INGV-ECHAM4 (Gualdi et al., 2008). Mean differences in the values of bio5, bio6, bio19, bio20 encountered in the SP range among classical 1950–2000 climatic layers, and these newly constructed rasters were, respectively, 0.601 °C pixel^{-1}, 0.225 °C pixel^{-1}, 12.18 mm pixel^{-1} and 0.217 °C pixel^{-1} (Appendix S2). Although the NC bioclimatic rasters do not perfectly represent climatic conditions experienced by the SP during 1997–2014, this procedure mainly aimed at depicting uncertainty associated with our climatic data by avoiding underestimation of climatic tolerances of SP and consequently minimizing omission errors in the predictions. We simulated the future potential distributions of SP in 2050 and 2070 using two different global climatic models (GCMs), that is the Hadley Centre Global Environmental Model version 2 HadGEM2-ES (Collins et al., 2011) and the Model for Interdisciplinary Research on Climate version 5 MIROC5 (Watanabe et al., 2010). We selected these GCMs because their predictions of future temperature and precipitation rank among the most reliable according to model evaluation procedures used in AR5 of

IPCC (Flato et al., 2013). For each GCM, we ran simulations using two different climatic datasets relative to the representative concentration pathways RCP4.5 and RCP8.5, which assume moderate and extreme global warming, respectively (Van Vuuren et al., 2011). Landscape data for Portugal were obtained from the CORINE land cover 2006 database (CEC, 1993) for the period 1990–2000. We used the distribution maps of three land cover categories at a resolution of 30 arc seconds: (1) pure conifer forests, (2) mixed conifer-angiosperm forests and (3) pure angiosperms forests (Fig. 1). All pixels not belonging to one of these three land cover categories were assigned to a uniform land cover category referred to as 'open lands' (Fig. 1).

Species distribution modeling

A principal component analysis (PCA) was first performed using the R package ADE4 (Dray & Dufour, 2007) to explore the spatial variability of climate in Portugal. We ran this PCA by considering all pixels of a 30-arc second raster of Portugal as individuals and 20 bioclimatic descriptors as variables and mapped the scores of each pixel on the two first principal components of the PCA. We used four SDM algorithms to model the distribution of SP: the generalized linear model with a binomial family (GLM), the classification and regression trees (CART), the boosted regression trees with a binomial family (BRT; Friedman et al., 2000; Elith et al., 2008) and the MAXENT algorithm based on the maximum entropy approach (Phillips et al., 2006) using the R packages DISMO (Hijmans et al., 2013), TREE (Ripley & Ripley, 2014) and GBM (Ridgeway & Ridgeway, 2004). To avoid the problem of separation in GLM with binary response (Heinze & Schemper, 2002), we used an approach based on a penalized likelihood correction to the standard binomial GLM score function in the R package GLMNET (Friedman et al., 2010). Eighty percentage of presences and absences from ABS1 dataset were used to calibrate models while the remaining 20 percentage of data were kept for model evaluation. All probability maps were converted into the presence/absence binary maps by implementing the 'lowest presence threshold' (LPT; Pearson et al., 2007) that minimizes the omission error. For each climatic scenario, we mapped the number of models predicting a presence in any pixel in Portugal and the Mediterranean basin (ensemble forecasting approach; see Araújo & New, 2007). In addition, for the current climatic conditions, we constructed a consensus binary prediction (C-prediction) by assigning a presence to each cell that was predicted suitable

for at least 75% of models. We constructed Multivariate Environmental Similarity Surface (MESS) maps that measure the dissimilarity between the climatic spaces of the training and projected areas (Elithn et al., 2010). The predictive power of each model was evaluated by calculating the area under the curve of the receiving operator curve (AUC; Fielding & Bell, 1997).

Modelling the spread of SP

Since the range of SP in 2002 was relatively well defined (Pimentel et al., 2006), we divided the presence dataset in two subsets, that is localities that were reached by SP before and after 2002 (Fig. 1). However, data were inadequate to accurately depict the range of SP for the remaining years, between 1997 and 2014. Still, we recognize that in 2002, the southern border of the SP range was not established with perfect exactitude, as SP nests were detected in the surroundings of Nazaré but were not precisely georeferenced. We calculated the ratio between the spreading distances (i.e. distance separating the area where SP was firstly detected in 1997 and the occurrences of SP situated at latitudinal extremes across its geographic range) and the spreading time for three periods: the 1997–2014, the 1997–2002 and the 2003–2014 periods. We used the MigClim model (Engler & Guisan, 2009) in MIGCLIM R package (Engler et al., 2012) to model the spread of SP. We ran numerous MigClim simulations by varying the values of the dispersal kernel (more than 115 combinations) to identify the parameters' combinations best explaining the current distribution of SP. The models maximizing the sum of sensitivity (i.e. true positive rate) and specificity (i.e. true negative rate) were considered as the most accurate. We did not consider the distribution of SP in 2002 when estimating the predictive power of MigClim models because the distribution data of SP in 2002 were relatively imprecise and the outbreak experienced by SP in 1997 probably inflated the initial spread rate of this population. A small patch of 2 km^2 within MNL corresponding to the first detection of SP larvae in 1997 (Pimentel et al., 2006) was considered as the source of the SP in MigClim simulations (Fig. 1). The consensus 'C-projection' map was used as the input binary map of climatic suitability. A dispersal step was generated each year, and a newly colonized cell was considered prone to generate dispersing propagules 1 year after colonization (iniMatAge = 1, PropaguleProd = 1; Engler et al., 2012). Even if long-distance dispersal events occurred during the spread of PPM in France (Robinet et al., 2012), our measures of SP spread suggest that

human-mediated dispersal events have not occurred for this population. We consequently did not consider long-distance dispersal events in models. The pure angiosperm forests were considered as barriers to dispersal ('weak barriers' to dispersal according to Engler et al. (2012). As preliminary results showed that the spread rate of SP was anisotropic (see below), we independently ran two set of MigClim simulations that accounted for northward and southward spread of SP and subsequently merged both sets of projections.

Results

The realized climatic niche of the SP

The first principal component of the PCA corresponded to a north–south climate gradient well depicted in Fig. 2(a, c). The second axis separated the Western coasts of Portugal in relation to moderate maximal temperatures (bio5) and low temperature seasonality (bio2, bio4, bio7; Fig. 2). All SDMs have significant predictive power displaying AUCs superior to 0.93. All models identified the summer temperatures as the variable best explaining the current range of SP. Most models predicted only a narrow band along the Atlantic coast as climatically suitable for the SP in Portugal (Fig. 3). In contrast, inland regions of Portugal were considered climatically unsuitable by all models (Fig. 3). Models globally predicted that the current climatic conditions encountered in most lowland regions of the Mediterranean basin, except some coastal areas in Morocco and Northern Spain, would be unsuitable for SP establishment (Fig. 3). Some lowland regions of the Mediterranean basin (coasts of Israel, Italy and Greece) were predicted as suitable by only one model (Fig. 3). Some models, but not all, predicted European mountainous regions as climatically suitable (Fig. 3). Caution is needed, however, when interpreting the SDM predictions for these elevated regions in Europe as these areas display climatic conditions different from our calibration dataset (MESS values < 0; Fig. 2c). Models calibrated with the NR bioclimatic rasters displayed similar predictions (Fig. S1 in Appendix S3). Depending on the GCM and the RCP considered in this study, the summer mean temperatures within the current geographic range of SP are expected to increase from 1.4 to 3°C by 2050 compared to the period 1950–2000 and to become similar to those encountered in the adjacent inland regions where the SP is currently absent (Fig. S2 in Appendix S3).

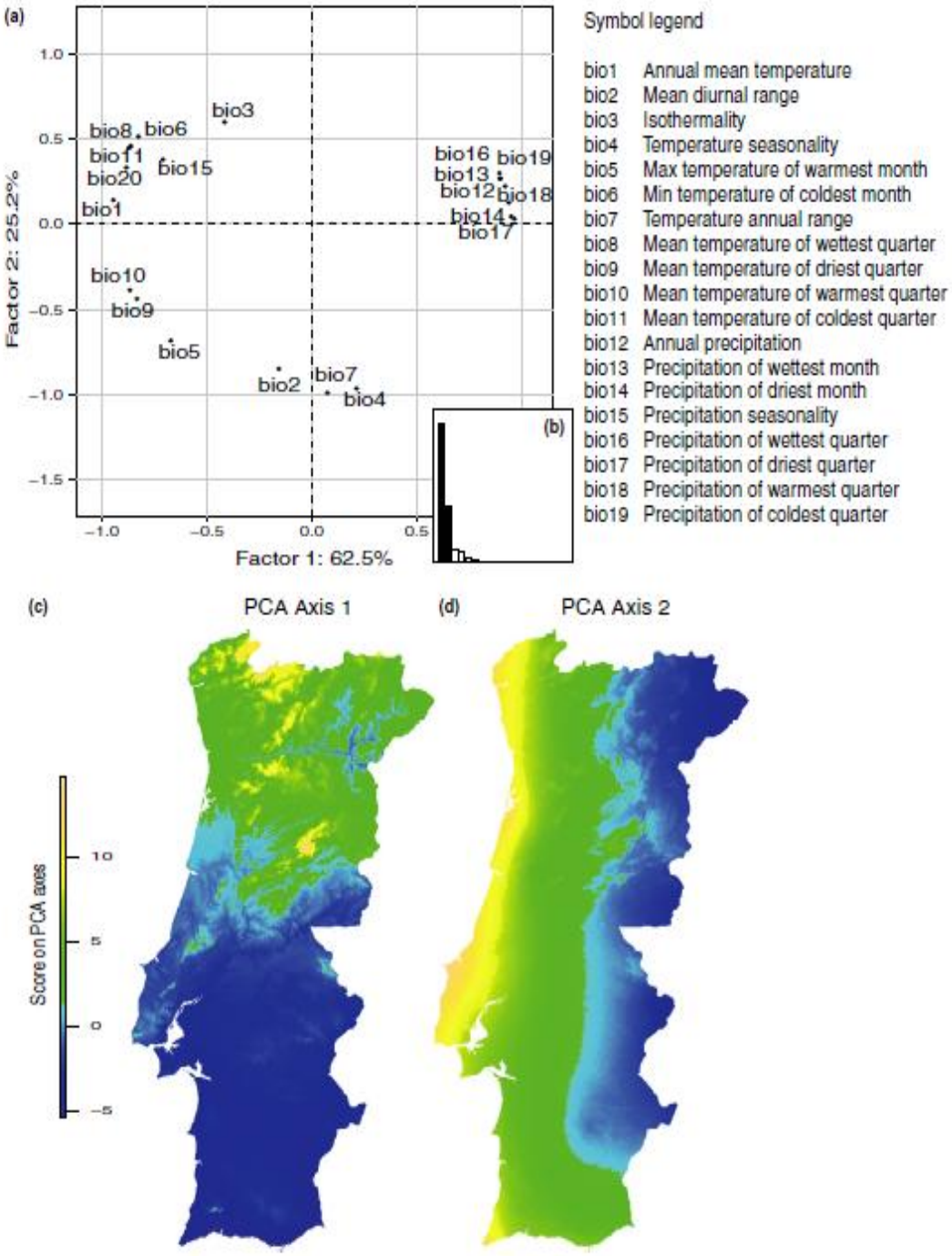

Figure 2 - Principal component analysis (PCA) performed on 20 bioclimatic variables extracted from all pixels in a raster of Portugal extracted from WORLDCLIM (Hijmans et al., 2005). Circle of correlation (a), eigenvalues diagram (b) and maps of scores of all pixels in Portugal on PCA axis 1 (c) and axis 2 (d) are represented.

Most models predicted that large parts of the current range of the SP might become unsuitable by 2050 and 2070 (Figs. 4 and S3 in Appendix S3). Most models predicted some coastal regions of Portugal (i.e. the surroundings of the towns of Peniche and Aveiro) to remain climatically suitable by 2050 and 2070 under the scenario 4.5 (Figs. 4 and S3 in Appendix S3). These trends were globally similar when calibrating SDMs with NR climatic layers (Figs. S2 and S4 in Appendix S3). However, the effects of global changes were predicted to be slightly more intense when calibrating SDMs with classical 1950–2000 climatic layers. The reduction of the extent of suitable climatic conditions in Portugal was predicted to be higher under the RCP 8.5 (Figs. 4 and S3 in Appendix S3). The Mediterranean basin, except for the extreme north-east of Spain and some mountainous regions, was predicted to globally remain climatically unsuitable by 2050 and 2070 (Figs. S5 and S6 in Appendix S3).

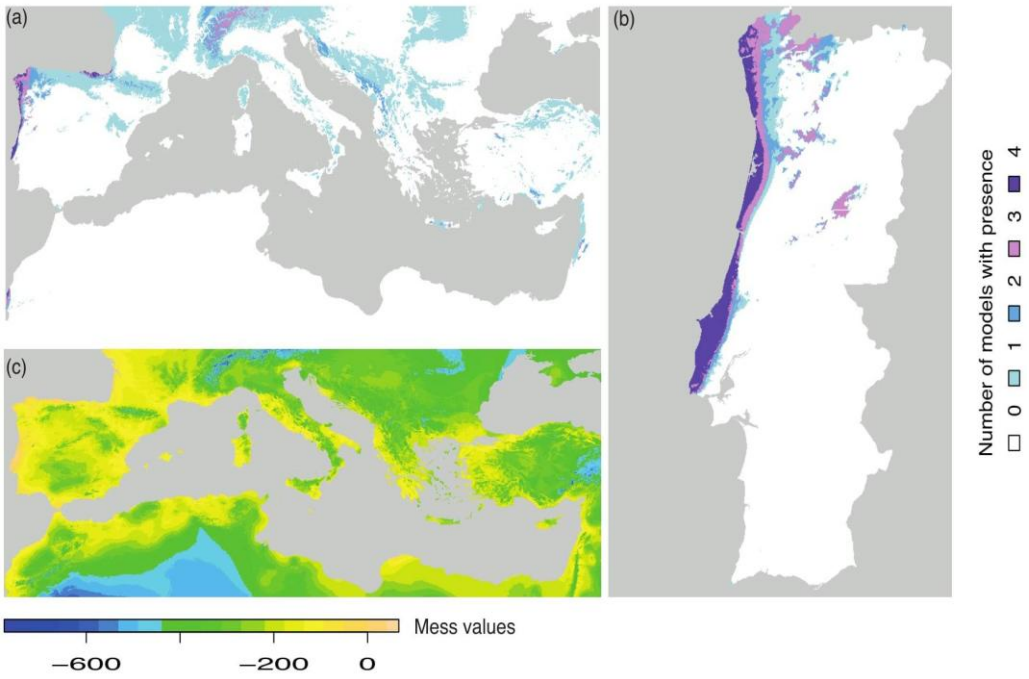

Figure 3 - Potential distribution of the summer population (SP) of *Thaumetopoea pityocampa* in (a) the Mediterranean basin and in (b) Portugal under current climate predicted by four bioclimatic models (boosted regression trees, generalized linear model, classification and regression trees, MAXENT) calibrated with variables extracted from WORLDCLIM (Hijmans et al., 2005). We mapped the number of models predicting a presence in these regions when implementing the lowest presence threshold (Pearson et al., 2007). (c) Multivariate environmental similarity surfaces (MESS maps).

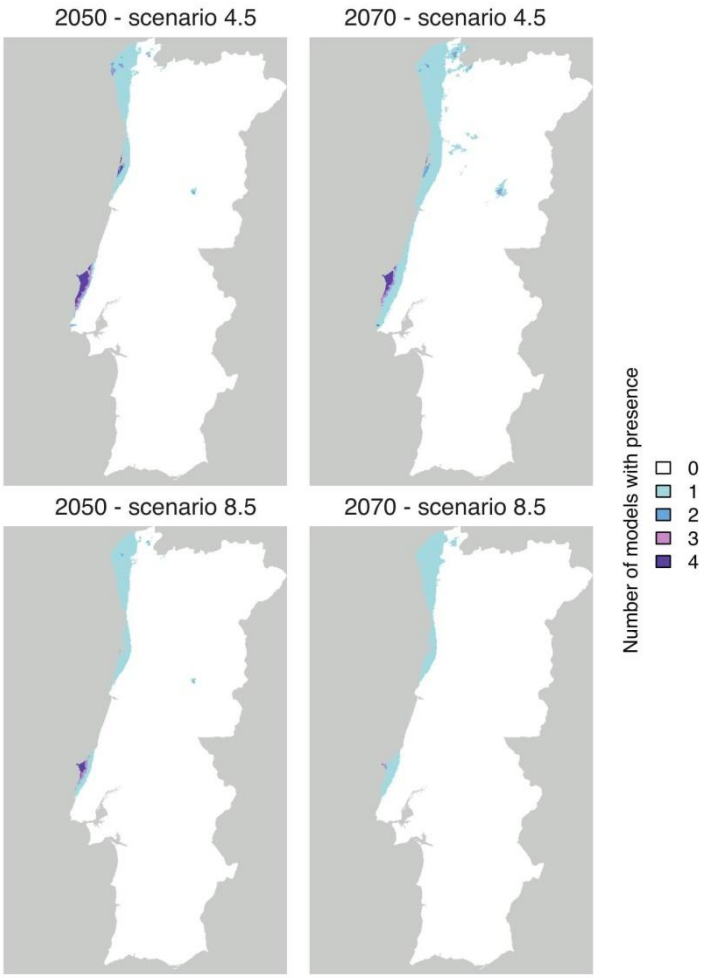

Figure 4 - Potential distribution of the summer population (SP) of *Thaumetopoea pityocampa* in Portugal 2050 and 2070 predicted by four bioclimatic models (boosted regression trees, generalized linear model, classification and regression trees, MAXENT) calibrated with variables extracted from WORLDCLIM (Hijmans et al., 2005). Future climate data were obtained from the Hadley Centre Global Environmental Model version 2 HadGEM2-ES (Collins et al., 2011). We predicted the future distribution of SP with two hypothetical scenarios of future greenhouse gas emissions (4.5 and 8.5). We mapped the number of models predicting a presence in these regions when implementing the lowest presence threshold (Pearson et al., 2007).

Modelling the spread of SP

The measured distances between the area where SP was firstly discovered in 1997, and both the southern and northern limits of its distribution in 2014, were, respectively,

42.69 and 28.25 km. Consequently, the estimates of southward and northward spread rates, respectively, yielded values of 2.51 and 1.66 km year^{-1} over the period 1997–2014. The estimates of the distances covered by SP southwards and northwards, during the period 1997–2002, yielded values of 16.90 and 12.25 km, respectively. The estimates of the northward rates of spread of SP in the periods of 1997–2002 and 2003–2014 yielded values of 2.5 and 1.36 km year^{-1}, respectively. The estimates of the southward rates of spread of SP in the periods 1997–2002 and 2003–2014 yielded values of 3.38 and 2.48 km year^{-1}, respectively. The parameters of the MigClim models best explaining the distribution of SP in 2014 are summarized in Table S1 (Appendix S4). The future range expansion of SP was predicted to remain confined to a narrow strip, along the Atlantic coast of Portugal (Fig. 5). Without considering future climatic changes, the SP was predicted to reach the district of Lisbon and the district of Aveiro in the centre region around 2050 (Fig. 5). Some regions in north Portugal were predicted as climatically suitable by 2050 or 2070 (Fig. 4) but out of reach of the SP with respect to landscape heterogeneity and dispersal constraints (Fig. 5).

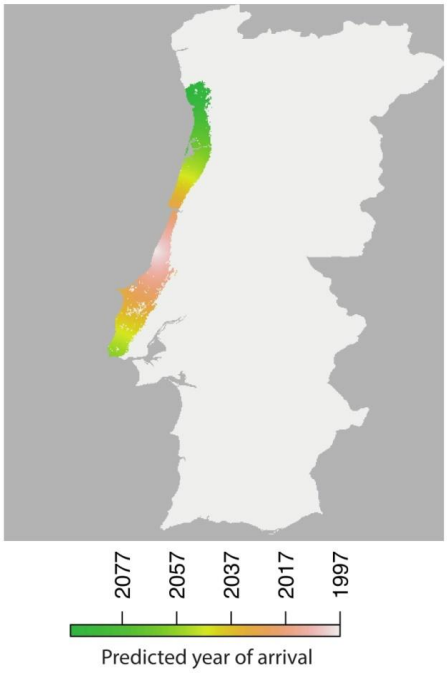

Figure 5 - Potential spread of the summer population (SP) of *Thaumetopoea pityocampa* in Portugal predicted by MigClim model. This projection was constructed by merging the outputs of two MigClim models that were calibrated independently to model the past spread SP northward and southward, respectively.

Discussion

A climatic niche shift induced by allochronic speciation

Allochronic speciation may induce geographic range shifts or adaptations (through phenotypic plasticity or evolutionary changes) in the populations that abruptly face new environmental conditions. Both phenomena have been observed in PPM. Thus, laboratory experiments have shown the presence of adaptations to high temperatures in SP larvae in comparison with classical WPs (Santos et al., 2011b). Yet, our study clearly demonstrates that the distribution of SP is strongly constrained by environmental parameters and only a subset of the geographic range of the classical WP (i.e. the Mediterranean basin) is currently climatically suitable for the long term establishment of the SP.

Considering that our models were calibrated with a majority of true absences that enhance the predictive power of bioclimatic models (Elith et al., 2010), the present study may be expected to accurately depict the niche of SP and provide reliable predictions. Despite intensive searching between 1997 and 2014, nests of SP have only been detected within a narrow strip along the Atlantic coast of the country, but never across inland regions.

The observed maximal distance of spread by SP during the period 1997–2014 (ca. 40 km) largely exceeds the distances separating some of the absence records situated in inland regions and the area where SP was first discovered (Fig. 1). Some absences were indeed recorded in locations where *P. pinaster* stands are distributed over large areas (Fig. 1), up to 10 km from the area where the SP was first discovered in MNL. Such observations strongly suggest that SP dispersal ability and host-related constraints alone cannot explain that expansion into inland regions did not take place. Therefore, climate can probably be identified as the most relevant factor explaining why the geographic range of SP has remained confined to part of the coastal regions of Portugal. In addition, density of SP nests (i.e. number of nests m^{-2}) gradually decreases between the coast and the inland regions of Leiria province (Godefroid, personal observation), which is consistent with the most parsimonious hypothesis that coastal regions are climatically more suitable for SP long-term establishment. With regard to reliability of our absences data, this study clearly demonstrates that a niche shift occurred during allochronic

speciation in PPM and proves the crucial importance of accounting for intraspecific diversity when calibrating SDMs for conservation, invasion and pest control purposes.

All models captured the average maximal summer temperature as the climatic variable presenting the highest contribution to discriminate our presence and absence data, confirming that tolerance of larvae to heat probably constitutes the main constraint relative to a shift in the phenology of PPM. Adaptations of SP to high temperatures (Santos et al., 2011b) are probably neither sufficient to allow for the expansion of SP into the inlands of Portugal nor into most regions of the Mediterranean basin. The extreme temperatures observed in the summer in most areas of the Mediterranean basin are indeed lethal for SP larvae (Santos et al., 2011b) and impede SP long-term establishment in these regions. Beyond summer heat, other climate characteristics such as winter and spring temperatures act as serious constraints upon the phenological shift of SP. The relative contributions of these climatic descriptors were, however, globally small in our correlative SDMs. It is generally expectable that relatively high spring temperatures should be required for SP reproduction, as mating takes place at night in May and cold weather may prevent adult activity. Regarding WPs, a threshold of 14°C below which WP females have low flight activity was identified by Battisti et al. (2006). However, Zhang & Paiva (1998) showed that temperatures above 22°C can hinder reproductive activity and that females first started calling when air temperatures dropped below that threshold. Low winter temperatures and high soil moisture are thought to cause high mortality to overwintering pupae of PPM in the soil (Markalas, 1989). However, in some cases, diapausing pupae of WP were observed to remain in the soil for over 8 years before emergence, suggesting that they are prone to resist cold winter temperatures (Aimi et al., 2006; Salman et al., 2016). Preliminary experiments showed that soil temperatures (at 10 and 20 cm depth) measured during the winter of 2008 in localities where SP is present, ranged from 5 to 14°C. In this context, outputs of some correlative SDMs might seem surprising as they predict high climatic suitability in mountainous areas, where very cold winter temperatures and fresh spring temperatures are common (Fig. 3). As these areas show a climate very different from the climatic space of the calibration dataset (see MESS maps in Fig. 3), our predictions for these areas have a low reliability and should be interpreted with caution (Elith et al., 2010). In this context, the question of how spring and winter temperatures interact to shape the geographic distribution of SP should be explored in depth to improve the reliability of the model predictions. Special features on Portuguese coast could explain

the successful establishment of the allochronic SP. Indeed, the climatic conditions encountered in this area are particular for Portugal and for Europe (i.e. oceanic climate with mild winters and moderately hot summers; Fig. 2) and have probably been little constraining with respect to the shift of the larval development period from winter to summer. Moreover, this region is characterized by elevated relative air humidity that perhaps may alter the tolerance of insects to extreme temperatures (Fields, 1992). The moderate temperatures and the high levels of relative humidity encountered in the summer across these coastal regions may interact to shape the current geographic distribution of the SP. Several phenology changes occurred during the evolutionary history of the Thaumetopoea genus (Santos et al., 2007; Simonato et al., 2013). One plausible hypothesis is that phenology is labile in Thaumetopoea species and that, consequently, other allochronically reproducing populations have diverged from the classical PPM in separate geographic regions but failed to get established over long term, presumably due to strong environmental constraints associated with phenological shift. For instance, in Greece, some adults of PPM were trapped in the spring by Athanassiou et al. (2007) but no other population of PPM with such abruptly shifted life cycle has ever been observed in the country. Still, further survey is needed to search for allochronic individuals that could recurrently occur in separate geographic regions and therefore confirm this hypothesis.

The geographic distribution of SP: the role of spread

The estimated rates of spread of SP over the period 1997– 2014, ranging from 1.66 to 2.51 km year^{-1}, were globally similar to those reported for WP by Démolin (1969) and Battisti et al. (2005). However, they were largely inferior to the rate of spread of PPM recorded in northern France, over the period 2000–2009 (ca. 5.6 km year^{-1}; Battisti et al., 2005) and laboratory-estimated dispersal capacity by Robinet et al. (2012). Our moderate estimates of SP spread rate suggest that human-mediated long dispersal events have probably not occurred during SP range expansion as reported in WP (Robinet et al., 2012).

The spread rate of SP has probably not been uniform across space and time since 1997. It appears that the SP spread faster over the period 1997–2002. This may be explained by the outbreak phase experienced by the SP in 1997, which has abruptly increased the population density and consequently raised the spread towards adjacent areas. The

subsequent diminution of the SP spread rate might be explained by the decrease in population densities typical of post-outbreak phases. We observed that SP spread was slightly faster southward (2.51 km $year^{-1}$) than northward (1.66 km $year^{-1}$). Two hypotheses may explain this trend. On the one hand, wind regimes might play a main role in driving dispersal of SP across Portugal. It has already been mentioned by Pimentel et al. (2006) that the direction of dominant winds in Leiria province during the period of adult flight of SP (i.e. mainly south-eastward) might explain the faster southward range expansion of SP. It must be noted the PPM females has poor flight capacities and as such their dispersal may be markedly affected by dominant winds. On the other hand, it is likely that landscape fragmentation influences SP dispersal as reported for other forest insects (Morin et al., 2009). In northern France, the PPM was shown to spread across large agricultural areas through the ecological continuities created by trees outside forests (mostly ornamental trees scattered across fields and towns; Rossi et al., 2016). Although we did not directly sample the distribution of trees outside forests in Leiria province, it is likely that the large number of pines planted in open areas have facilitated the spread of SP southward (see 'open lands' in Fig. 1). However, field data are currently lacking, preventing a formal integration of these elements in our models.

What future for the SP?

Effects of global change are predicted to be particularly harmful for small-range organisms such as SP (Ohlemüller et al., 2008). The present survey corroborates this trend as most of the SDMs predicted SP to be endangered following ongoing environmental changes. Within the current SP range, the average summer temperatures are expected to increase from 1.4 to 3°C by 2050, depending on the scenario considered and become similar to those recorded in areas where SP is currently absent (Fig. S2 in Appendix S3). Moreover, the limited dispersal abilities of SP will probably enhance the detrimental effect of global change on this population. Some areas of northern Portugal are predicted to be climatically suitable for SP by 2050 but out of reach by natural dispersal (Figs. 4 and 5). These trends highlight the importance of coupling SDM with dispersal and demographic models when assessing the future distribution of species (Engler & Guisan, 2009). We cannot exclude the possibility that SP might be introduced and become established in other regions of Portugal or of the Mediterranean basin

through human-mediated long dispersal events, as it has been the case for the WP (Robinet et al., 2012). However, this hypothesis is unlikely for two main reasons: (1) importations of pines from Leiria province are currently prohibited by authorities of many countries because of the recent detection of the pine wood nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) in forests of Portugal (Branco et al., 2014); (2) the climate of most areas of the Mediterranean basin is predicted as unsuitable for long-term establishment of the SP (Fig. 3, Figs. S5 and S6, Appendix S3). The reliability of SDM predictions must be considered with caution as these correlative tools mainly depict the realized niche of a species (i.e. inferred from its geographic distribution) rather than its fundamental niche (i.e. reflecting its physiological tolerances; Soberón & Peterson, 2005). Moreover, SDMs usually do not anticipate adaptations of species to environmental changes and implicitly assume niche conservatism across space and time. However, niche shifts are not uncommon and examples of adaptation to new environmental conditions by phenology shifts (Parmesan & Yohe, 2003) or increased thermal tolerances (Hill et al., 2013) have been reported. There is thus an urgent need to model the adaptive potential of SP to new environmental conditions to allow proper assessment of its future distribution. Although uncertainty is inherent to SDM predictions, the trends presented in this study are clear and suggest that SP should be added to the long list of threatened organisms from ongoing global changes.

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Supporting information

Appendix S1 - Distribution of SP assessed with a field survey performed at the end of the summer of 2014.

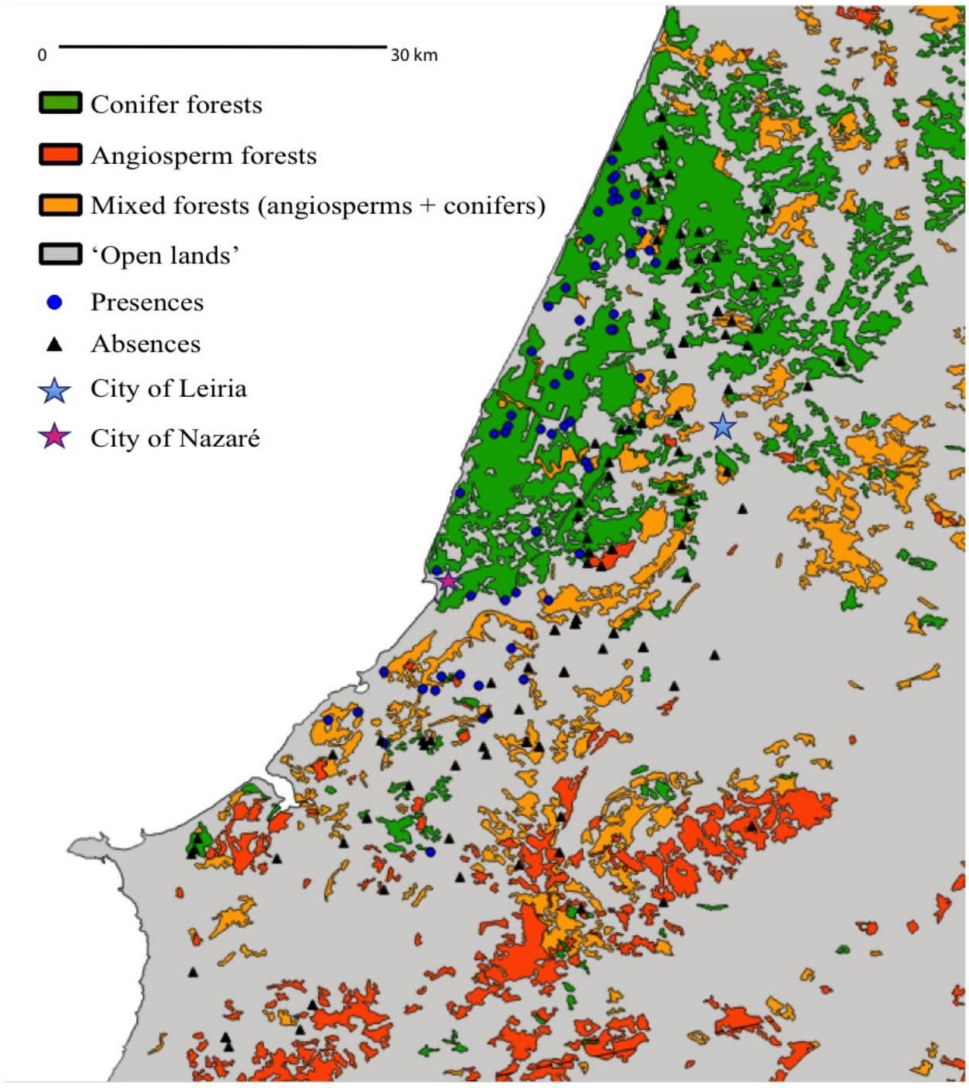
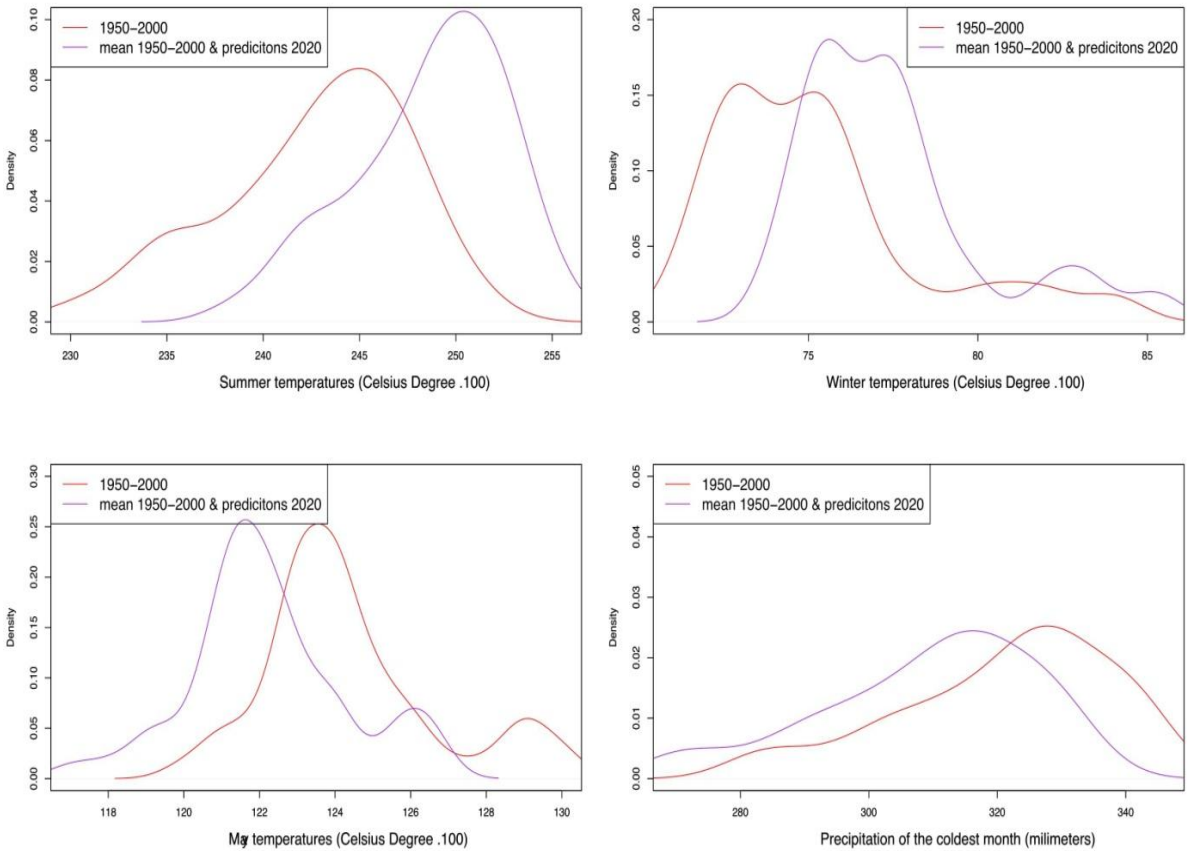


Figure S1 - Distribution of SP according to a sampling survey performed in 2014. Distribution of SP was assessed by inspecting pine stands in a *circa* 500m-radius circle in 131 sampling stations represented by circles and triangles in the map.

Appendix S2 - Comparison of bioclimatic variables extracted from Worldclim (period 1950–2000) with newly constructed climatic variables (NR rasters) in Portugal.



Figures S1-S2 - Values of the climatic variables extracted from (1) WorldClim layers relative to 1950-2000 period and (2) newly constructed layers relative to the mean between predictions of INGV-ECHAM4 climatic model by 2020 (A2 scenario) and the 1950-2000 Worldclim climatic layers.

Appendix S3 - Prediction of the potential distribution of SP in Portugal and in the Mediterranean basin under current and future climatic conditions.

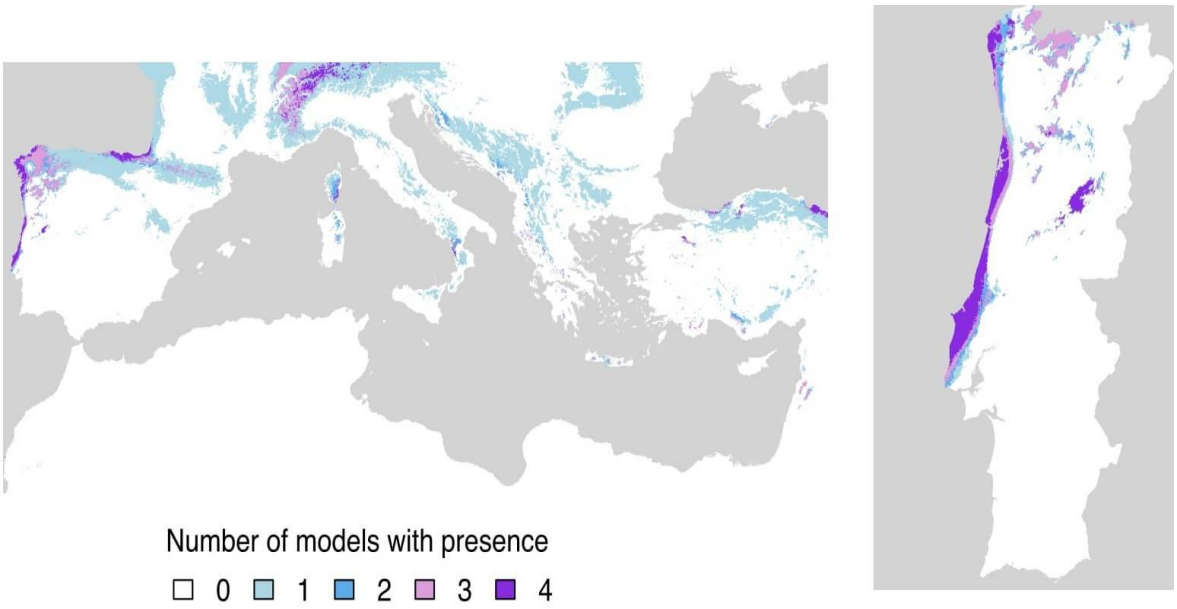


Figure S1 - Potential distribution of the summer population (SP) of *Thaumetopoea pityocampa* in Portugal and in the Mediterranean basin predicted by four bioclimatic models (boosted regression trees, generalized linear model, classification and regression trees, MaxEnt). Models were calibrated with four climatic variables (mean temperature of the warmest month, mean temperature of the coldest month, mean temperature of May, precipitation of the coldest quarter) extracted from the newly constructed climatic variables in Portugal, by averaging the 1950-2000 Worldclim climatic layers with layers corresponding to predictions of future climate by 2020, under the A2 scenario INGV-ECHAM4 (Gualdi *et al.*, 2008) used in the Fourth Assessment Report (AR4) of the Intergovernmental Panel on Climate Change (IPPC).

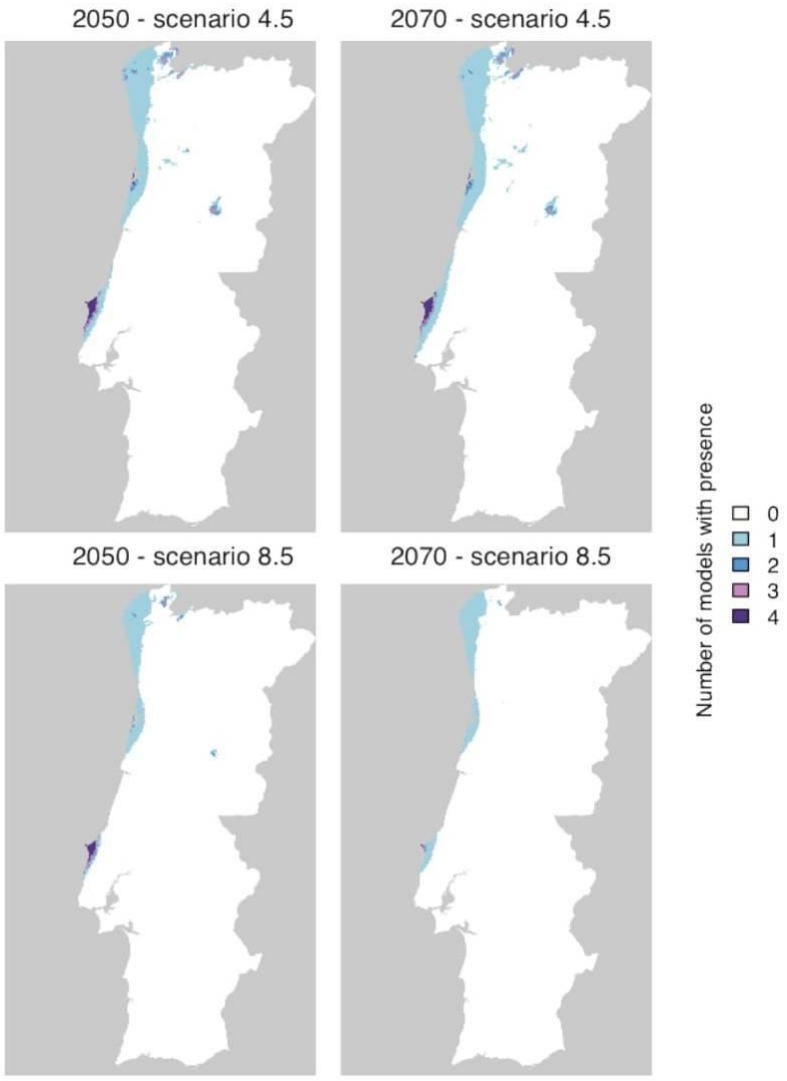


Figure S2 - Changes in temperatures within the geographic range of the summer population (SP) of *Thaumetopoea pityocampa* in Portugal predicted by global climate models by 2050 and 2070. Plots (a) and (b) show the changes in temperatures predicted by GCMs, respectively by 2050 and 2070, in comparison with the climate observed at occurrence points of SP extracted from the Worldclim data base (climatic condition for 1950-2000). Plots (c) and (d) show the changes in temperatures predicted by GCMs respectively by 2050 and 2070, in comparison with the climate observed at occurrence points of SP extracted from the newly constructed layers relative to the mean between predictions of INGV-ECHAM4 climatic model by 2020 (A2 scenario) and the 1950-2000 Worldclim climatic layers.

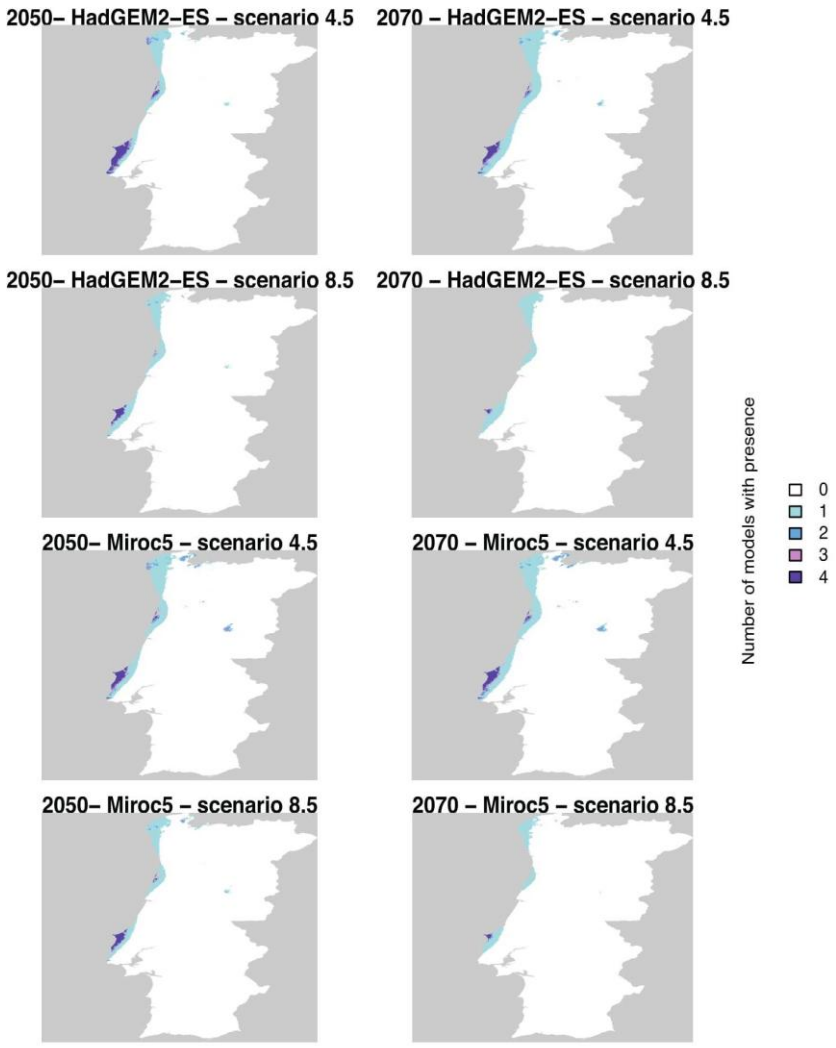


Figure S3 - Potential distribution of the summer population (SP) of *Thaumetopoea pityocampa* in Portugal in 2050 and 2070, predicted by four bioclimatic models (boosted regression trees, generalized linear model, classification and regression trees, MaxEnt). These models were calibrated with four climatic variables (mean temperature of the warmest month, mean temperature of the coldest month, mean temperature of May, precipitation of the coldest quarter) extracted from the Worldclim database (Hijmans *et al.*, 2005) that represent climatic trends during the period 1950-2000. Future climate data were obtained from the Model for Interdisciplinary Research on Climate version 5 MIROC5 (Watanabe *et al.*, 2010). We predicted the future distribution of SP with two hypothetical scenarios of future greenhouse gas emissions (4.5 and 8.5). We mapped the number of models predicting the presence of SP in these geographic regions. For each model, binary maps of presence/absence were constructed using the lowest presence threshold (Pearson *et al.*, 2007).

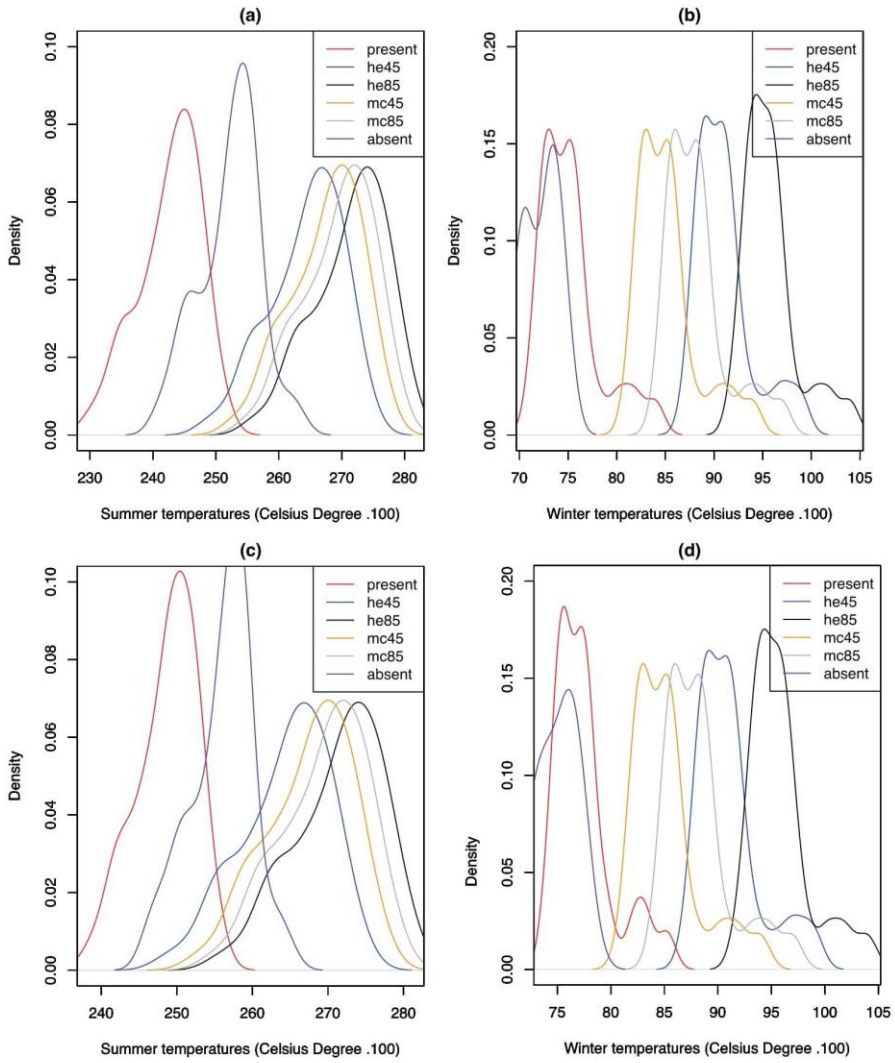


Figure S4 - Potential distribution of the summer population (SP) of *Thaumetopoea pityocampa* in Portugal in 2050 and 2070 predicted by four bioclimatic models (boosted regression trees, generalized linear model, classification and regression trees, MaxEnt). These models were calibrated with four climatic variables (mean temperature of the warmest month, mean temperature of the coldest month, mean temperature of May, precipitation of the coldest quarter) extracted from the newly constructed climatic variables in Portugal that were constructed by averaging the 1950-2000 Worldclim climatic layers with layers corresponding to predictions of future climate by 2020 under the A2 scenario INGV-ECHAM4 (Gualdi *et al.*, 2008) used in the Fourth Assessment Report (AR4) of the Intergovernmental Panel on Climate Change (IPPC). Future climate data were obtained from the Model for Interdisciplinary Research on Climate version 5 MIROC5 (Watanabe *et al.*, 2010) and from the Hadley Centre Global Environmental Model version 2 HadGEM2-ES (Collins *et al.*, 2011). We predicted the future distribution of SP with two hypothetical scenarios of future greenhouse gas emissions (4.5 and 8.5). We mapped the number of models predicting a presence in these geographic regions. For each model, binary maps of presence/absence were constructed using the lowest presence threshold (Pearson *et al.*, 2007).

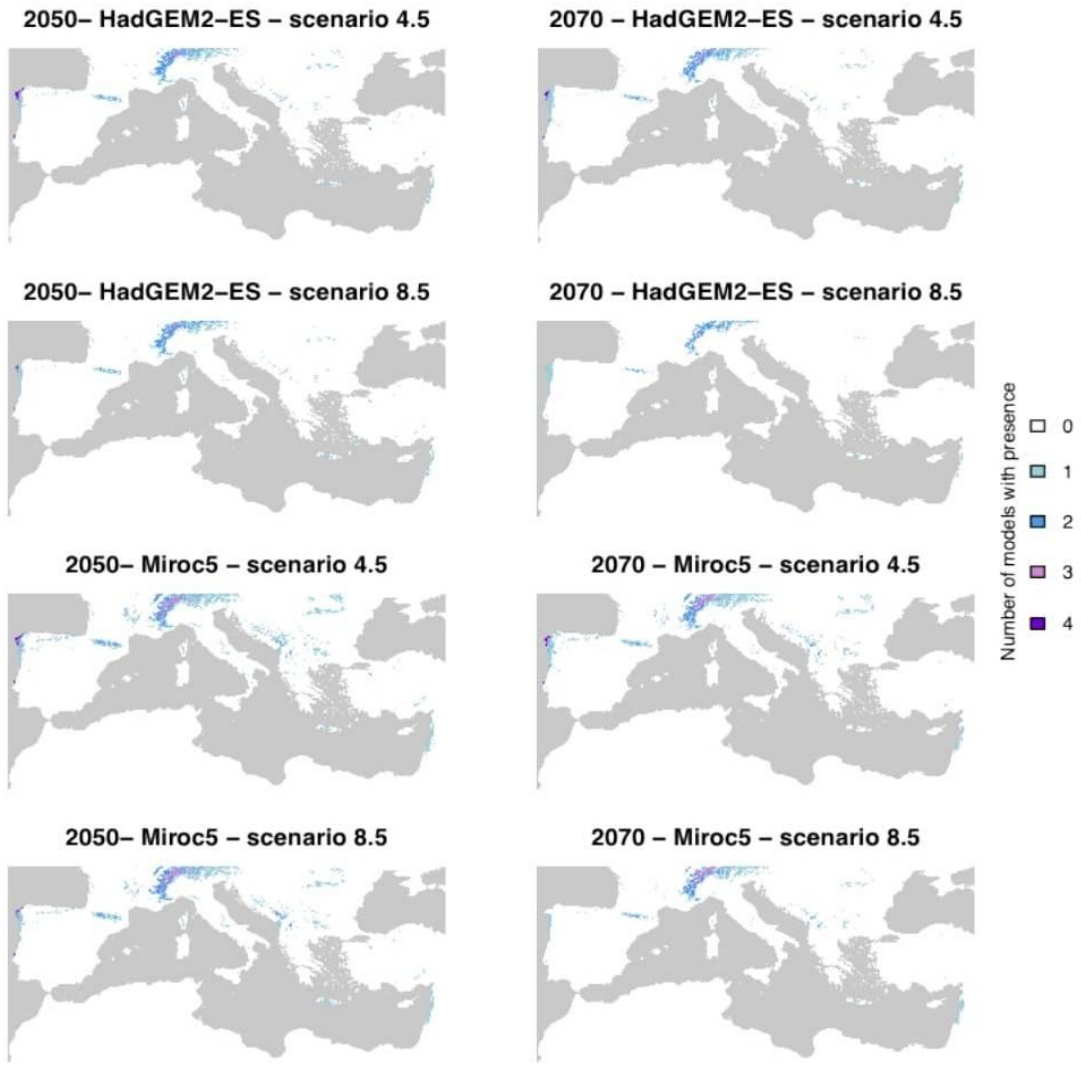


Figure S5 - Potential distribution of the summer population (SP) of *Thaumetopoea pityocampa* in the Mediterranean basin in 2050 and 2070 predicted by four bioclimatic models (boosted regression trees, generalized linear model, classification and regression trees, MaxEnt). These models were calibrated with four climatic variables (the mean temperature of the warmest month, the mean temperature of the coldest month, the mean temperature of May, precipitation of the coldest quarter) extracted from the Worldclim database (Hijmans *et al.*, 2005) that represent climatic trends during the period 1950-2000. Future climate data were obtained from the Model for Interdisciplinary Research on Climate version 5 MIROC5 (Watanabe *et al.*, 2010) and from the Hadley Centre Global Environmental Model version 2 HadGEM2-ES (Collins *et al.*, 2011). We predicted the future distribution of SP with two hypothetical scenarios of future greenhouse gas emissions (4.5 and 8.5). We mapped the number of models predicting a presence in these geographic regions. For each model, binary maps of presence/absence were constructed using the lowest presence threshold (Pearson *et al.*, 2007).

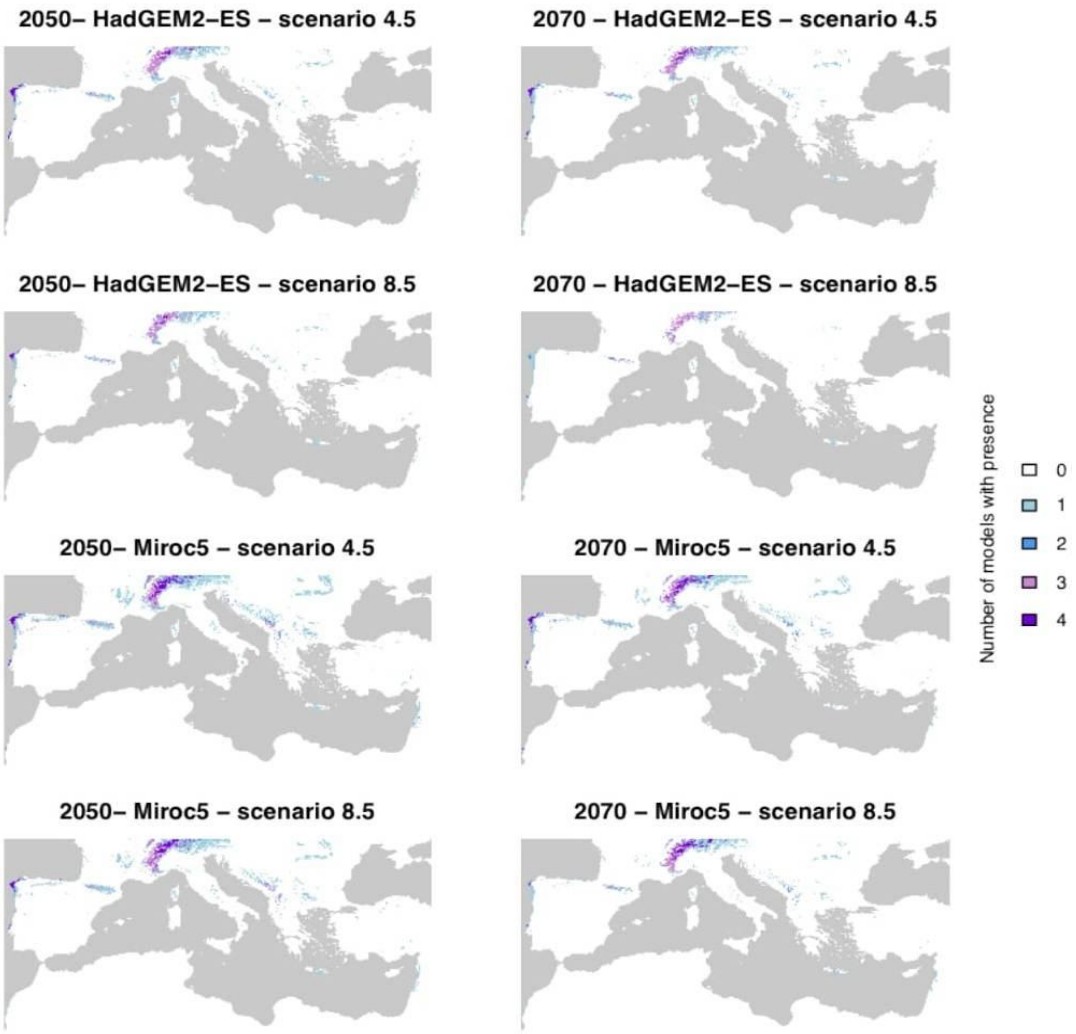


Figure S6 - Potential distribution of the summer population (SP) of *Thaumetopoea pityocampa* in the Mediterranean basin in 2050 and 2070 predicted by four bioclimatic models (boosted regression trees, generalized linear model, classification and regression trees, MaxEnt). These models were calibrated with four climatic variables (mean temperature of the warmest month, mean temperature of the coldest month, mean temperature of May, precipitation of the coldest quarter) extracted from the newly constructed climatic variables in Portugal that were constructed by averaging the 1950-2000 Worldclim climatic layers with layers corresponding to predictions of future climate by 2020 under the A2 scenario (INGV-ECHAM4; Gualdi *et al.*, 2008) used in the Fourth Assessment Report (AR4) of the Intergovernmental Panel on Climate Change (IPPC). Future climate data were obtained from the Model for Interdisciplinary Research on Climate version 5 MIROC5 (Watanabe *et al.*, 2010) and from the Hadley Centre Global Environmental Model version 2 HadGEM2-ES (Collins *et al.*, 2011). We predicted the future distribution of SP with two hypothetical scenarios of future greenhouse gas emissions (4.5 and 8.5). We mapped the number of models predicting a presence in these geographic regions. For each model, binary maps of presence/absence were constructed using the lowest presence threshold (Pearson *et al.*, 2007).

Appendix S4 - Parameters of the MigClim models best explaining the southward and northward spread of SP between 1997 and 2014.

Table S1 - Parameters of Miglcim model that best explain the range expansion) of the summer population (SP) of *Thaumetopoea pityocampa* in Portugal, observed between 1997 and 2014.

Range expansion direction	PropaguleProd	IniMatAge	DispKernel	Sum of model sensitivity and specificity
North	1	1	c (0.6, 0.3)	1.84
South	1	1	c (0.7,0.5)	1.54

CHAPTER 2

Spatio-temporal population structure in a context of allochronic differentiation

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To submit

Spatio-temporal population structure in a context of allochronic differentiation

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Abstract

Allochrony refers to a situation where reproductive isolation is driven by phenological changes. It can lead to population differentiation and even to a speciation process, either directly or as a reinforcement factor. It has recently been suggested to be more widespread than previously considered. A promising case study was discovered in the pine processionary moth, *Thaumetopoea pityocampa*, a Mediterranean defoliator of *Pinus* species. In Portugal, in and near the Mata Nacional Leiria (MNL), a unique population with a shifted phenology, known as the summer population (SP), co-occurs with a population following the typical life cycle, known as the winter population (WP). Reproduction periods of both populations are not synchronized, and the SP is highly differentiated. The objectives of the present study were to characterize the sympatric SP and WP over the whole SP distribution range using a systematic sampling along two transects as well as a population study at a larger geographical scale. We found a strong population structure of the species over Portugal, with a significant pattern of isolation by distance. We also showed that the SP was highly differentiated from all other sampled populations. Results obtained from the males sampled along two transects in 2012 and 2014 showed no within-population differentiation, suggesting a high stability over time. We also evidenced that SP phenology was more plastic than expected, with a limited number of SP males emerging each year at the beginning or the end of the WP season, which could allow hybridization events. In the meantime, we only identified very few introgressed individuals, which suggest that gene flow is nonetheless highly reduced. Interestingly, the spatial distribution of the two populations showed contrasting patterns, the SP being mostly limited to the sea shore where the WP is scarce or absent, which could suggest competitive exclusion. Once clearly occurring in a sympatric

context, the allochronic differentiation tends to develop nowadays in parapatry when fine scale patterns are considered.

Keywords: allochronic speciation, microsatellites, phenology, reproductive isolation, *Thaumetopoea pityocampa*

Introduction

Speciation is nowadays recognized as a dynamic process in which the respective role of forces driving ecological differentiation and reproductive isolation can change trough time and space. Speciation processes were primarily categorized based mostly on the geographical context (from allopatry to sympatry). It is however now largely admitted that other factors, either intrinsic or extrinsic, can be the primary drivers of speciation and modulate its scenarios and pathways (Kirpatrick & Ravigné, 2002; Butlin et al., 2008; Schluter & Conte, 2009; Kisel & Barraclough, 2010; Serviedo et al., 2011; Abott et al., 2013). Recent studies have tentatively developed a unified framework for studies on speciation (Kirpatrick & Ravigné, 2002; Dieckmann et al., 2004; Mullen & Shaw, 2014). Yet, detailed knowledge on case-specific events are needed to reveal the idiosyncratic aspects of speciation, in particular to describe their characteristics in time and space.

One powerful but overlooked mechanism possibly leading to differentiation is allochrony, a situation where reproductive isolation is due to a shift in reproductive time, which can be seen as an automatic magic trait (Serviedo et al., 2011). Allochrony can either initiate the speciation process or act as a reinforcement factor; it can be consecutive to adaptive differentiation (to climate or host use) or on the contrary pre-date and facilitate secondary adaptations (Taylor & Friesen, 2017). Interactions between its intensity and other differentiated traits under a peculiar spatio-demographic scenario will determine the level of gene flow and the completion of speciation. Allochronic speciation has so far received little attention as it was thought to be uncommon in nature (Pimentel et al., 2006; Santos et al., 2007; Yamamoto & Sota, 2009; Taylor & Friesen, 2017). However in the last years, several studies pointed allochrony as a contributor to divergence. Taylor & Friesen (2017) recently compiled a review of the role of allochrony in speciation and proposed that allochrony may be more widespread than

previously thought. The role of allochrony in the first step of the differentiation process can however be difficult to document.

One of the best-documented cases that satisfy the criteria proposed by Taylor & Friesen (2017) for true allochronic speciation is the pine processionary moth (PPM), *Thaumetopoea pityocampa* Denis & Schiffermüller (Lepidoptera: Notodontidae), in which a phenologically shifted population was identified in Portugal as being highly differentiated (Santos et al., 2007; 2011a). The PPM is a well-known defoliator occurring in all Mediterranean countries (Kerdelhué et al., 2009), causing damages in several *Pinus* species. This species has one year life cycle: in Portugal, adults emerge and reproduce in the summer (end of July to mid-September) and larval development occurs during fall and winter (October to end of February). The larvae then bury in the soil for pupation until adult emergence the following summer. This univoltine life cycle including larval development in the winter is typical for the PPM, and corresponds to the "winter phenology". In contrast, a unique population was discovered in 1997 in Portugal in a pure maritime pine (*Pinus pinaster* Ait.) forest located in the Mata Nacional Leiria (MNL) and does not follow the typical life cycle. This peculiar population was characterized by a "summer phenology", as adult emergence and reproduction took place in spring, from mid-April to early July, and larval development was completed in summer (Pimentel et al., 2006). It will hereafter be referred to as the "summer population" (SP) as opposed to all other known "winter populations" (WPs). It is noteworthy that the SP co-occurs in the MNL with a local WP that will be called the Leiria WP (Pimentel et al., 2006; Santos et al., 2007). This population has been studied for more than a decade, and previous results showed that the SP was genetically differentiated from the WP based on nuclear markers, while the two species shared a main mitochondrial haplotype (Santos et al., 2007; 2011a; Burban et al., 2016), suggesting a highly reduced level of gene flow. Field and laboratory studies conducted several years to characterize the adult activity of both populations showed that SP and WP flight periods (described as the curves of male trapping over time) did not overlap, which tended to confirm that the two populations had distinct reproductive periods (Santos et al., 2007, 2011a; Branco et al., 2016).

Recent field surveys showed that the distribution of the SP (based on nest localization) now covers a 120 x 20 kms square including the MNL where it was first discovered (Godefroid et al., 2016). Monitoring of adult emergence with pheromone traps coupled with genetic assignments allowed some striking observations (Burban et al., 2016). (i)

Six of the males trapped during the WP adult emergence period proved to belong to the SP, and were called "LateSP" individuals, all found within the MNL. This result suggested that the time of adult activity could partially overlap between the two populations. (ii) Some individuals proved to result from recent hybridization events between populations. In particular, a F1 hybrid was found at the southern edge of the SP range, and was trapped during the expected gap between the SP and the WP emergence periods. Altogether, these results suggested that the date of emergence alone could be misleading to determine if any trapped individual belonged to the SP or the WP. They also showed that reproductive isolation was incomplete between the allochronic populations.

In the previous works mentioned above, the SP was never sampled over its whole distribution range, but was rather studied in few sites along the coast. In particular, very few males were genotyped from the range limits, and the easternmost area was never included. Moreover, as only a small fraction of the trapped males were genetically assigned, we cannot at present provide a clear picture of the spatio-temporal distribution and diversity of this peculiar population, of the sympatric WP and their potential hybrids. Built on the knowledge gained from previous works and on robust procedures to detect hybrids and LateSP, the present study aims at characterizing the SP in a wider geographical context, and at providing for the first time detailed information over its whole distribution range. We relied on a design including two parallel transects to systematically trap and characterize males caught during both the SP and the WP flight periods. This design allowed to (i) test whether hybrids and LateSP were consistently identified and whether they occurred only in particular regions; (ii) relate the phenotypes (using the date of trapping as a proxy) to genetic assignments; (iii) explore the fine scale co-occurrence patterns of the apparently sympatric SP and WP over the SP range.

Material and methods

Sampling design

Pheromone traps were set up in 2012 and 2014 along two transects within the documented distribution of the SP in the Leiria region following Godefroid et al. (2016). Both transects were parallel, ca. 100 km long and positioned 20 km from each other. The first one corresponded to sites chosen along the sea shore and will be

hereafter named "coastal transect", while the second was positioned close to the eastern limit of the SP and will be referred to as the "inner transect" (Fig. 1). We used two traps per site, separated by a distance of about 10 meters. The pheromone lures were replaced every 6 weeks, following the methodology described in Santos et al. (2007, 2011a). The traps were installed in mid-April each year, and checked fortnightly during the entire period of adult flight of the two populations, i.e., until the end of September (Table S1, Table S2, Supporting information)

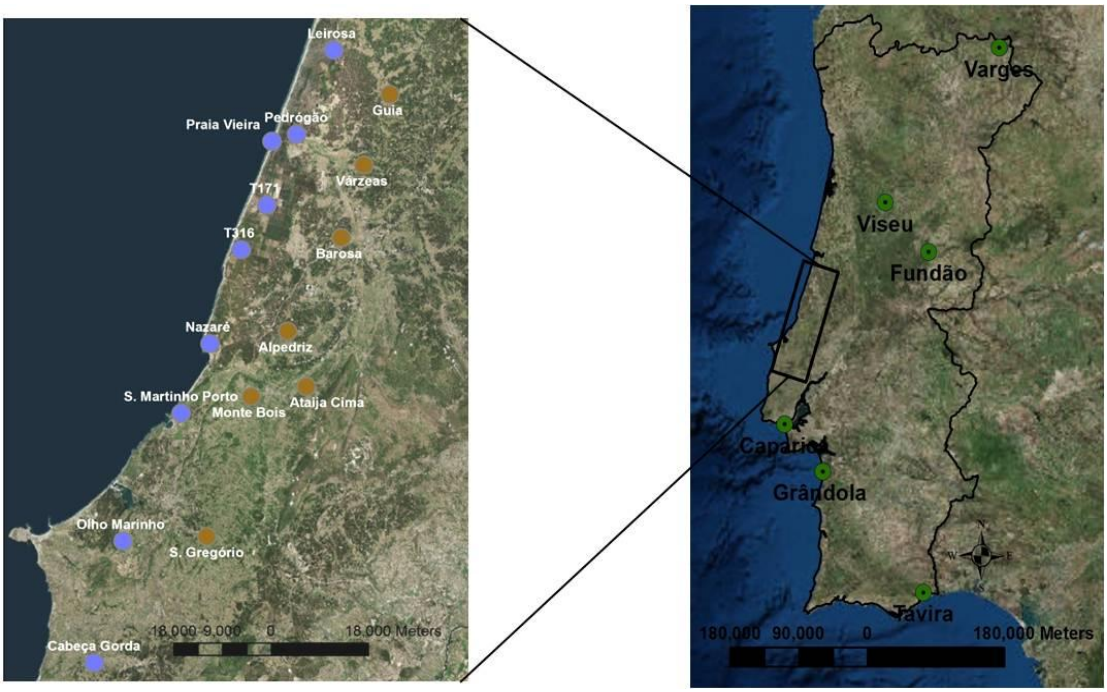

Figure 1 – Sampling sites of *T. pityocampa* over Portugal with Leiria zone highlighted (right) and in the two transects within the SP range (left). Blue and brown dots correspond to the coastal and inner transects, respectively.

Sampled individuals were kept in 95% ethanol, and classified *a priori* as belonging either to the SP or the WP populations according to their date of capture: individuals caught before mid-July were *a priori* coded as SP and after the end of July as WP.

Populations were also sampled from 6 localities over Portugal. In Fundão, Caparica, Grândola and Tavira, we used pheromone traps to catch males from mid-April to September as described above (Fig. 1, Table S1, Supporting information). In Varges, we sampled L1 larvae from 26 nests in *Pinus pinaster*, while the larvae from Viseu were those from Burban et al. (2016). All sampled individuals were immediately stored in 95% ethanol and kept at -18ºC until genetic analysis.

Microsatellite genotyping

A fraction of the males sampled in each site was genotyped using 20 microsatellite markers. We used 24 to 30 individuals sampled in each of the 6 localities over Portugal; note that all individuals caught in those sites showed a winter phenology (see Results). Concerning the two transects in the Leiria region, we used a sub-sample of males from each site and each date of collect over the whole trapping season both in 2012 and 2014 (see Table S2 for details). In some cases however, the trapped individuals could not be used for further genetic analyses due to a bad preservation of the samples due to predation (in particular, all samples from Leirosa in 2012, and some WP samples from Praia Vieira in 2012).

DNA was extracted from thoracic muscles for the males and from the whole body for larval samples using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturers protocol for animal tissue. DNA was eluted in 100 µl of AE buffer, and its concentration and quality were assessed using a NanoDrop spectrophotometer. PCR amplifications were performed using the Multiplex PCR Kit (Qiagen) and products were run and detected on an ABI 3500XL Genetic Analyzer (Applied Biosystems) in the GenSeq technological platform (Montpellier). Allele sizes were scored using the size standard GeneScan-500 LIZ (Applied Biosystems) and sized using the GeneMapper 5.1 software (Applied Biosystems).

Twenty microsatellite were used to genotype the sampled individuals, namely MS-*Thpit01*, MS-*Thpit03*, MS-*Thpit04*, MS-*Thpit05*, MS-*Thpit06*, MS-*Thpit07*, MS-*Thpit10*, *Ppit09*, *Ppit20*, *Ppit30*, *Ppit31*, *Ppit33*, *Ppit34*, *Ppit35*, *Ppit38*, *Ppit39*, *Ppit43*, *Ppit46*, *Ppit47*, *Ppit48*. Detailed molecular protocols for amplification and genotyping are described in Rousselet et al. (2004), A'hara et al. (2012) and Sauné et al. (2015).

Data analyses

Data were analyzed in two steps. We first used the whole data set, i.e., the 6 Portuguese populations together with the males sampled along the two transects in 2012 and 2014. In that case, we grouped the individuals caught in the Leiria region according to their phenology and year of capture, and considered Leiria WP 2012, WP 2014, SP 2012 and SP 2014. In a second step, we focused on the two gradients and analyzed separately the individuals trapped in each site at different dates, both in 2012 and 2014. For each locus and population from the whole data set, deviations from Hardy-Weinberg equilibrium

(HWE) were tested with 1000 permutations and 100 000 steps in the Markov chain, while linkage-disequilibrium (LD) was tested for all pairs of loci with 10 000 permutations, both using ARLEQUIN 3.11 (Excoffier et al., 2005). Expected and observed heterozygosities were estimated using the same software. The proportion of null alleles was estimated for each locus and population using the FREENA package (Chapuis & Estoup, 2007), following the Expectation Maximization (EM) algorithm of Demster et al. (1977). All file format conversions were done using CONVERT (Glaubitz, 2004) software.

a) Population genetic structure:

Population structure was first analysed through pairwise *Fst* either estimated directly or using the excluding null alleles (ENA) correction implemented in FREENA to correct for the positive bias induced by the presence of null alleles (Chapuis & Estoup, 2007). The 95% confidence intervals (CIs) were obtained by bootstrapping 1000 times over loci. Principal Component Analyses (PCA) were also performed to analyze population genetic structure of the microsatellite data sets, using the R package adegenet 1.4-2 (Jombart, 2008). Isolation by distance was tested over Portugal and within transects by testing the regression between *Fst* / (1-*Fst*) and the linear geographic distance between sites, and between *Fst* / (1-*Fst*) and the logarithm of geographic distance, following Rousset (1997).

Individuals were assigned to genetic clusters using a Bayesian inference method implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000). The number K of clusters varied from 1 to 12 when analyzing the whole data set, and from 1 to 10 for the transects. We used 50 000 burn-in steps followed by 50 000 Markov Chain Monte Carlo (MCMC) simulation steps with a model allowing admixture. To assess the consistency of results, we performed 10 independent runs for each value of K and compared the obtained individual Q-values. The number of K that provided the best fit to each data set was chosen by examining the curve of Log P(X|K) and using the ΔK method described in Evanno et al. (2005) and implemented in Structure Harvester (Earl & von Holdt, 2012).

b) Hybrid detection:

We took advantage of the procedure used in Burban et al. (2016) to detect potential hybrids. Briefly, from STRUCTURE (STR) analysis of the transect (SP and WP) data set,

we retrieved for each locus the allele frequencies corresponding to the SP and WP clusters to generate parental simulated data sets (1000 parental simulated SP individuals (pSPsim) and 1000 simulated WP individuals (pWPsim)). We then used these simulated parents to generate 1000 individuals for each hybrid category (first and second generation of hybrids, namely F1sim and F2sim, as well as backcross SP and WP, namely bcSPsim and bcWPsim) with Hybrid Lab (Nielsen et al., 2006). The complete simulated data set was analyzed with INTROGRESS, INT (Gompert & Buerkle, 2010) to determine the range of hybrid index of each category and with NewHybrid, NH (Anderson & Thompson, 2002) from which we calculated the performances in detecting each category (Vähä & Primer, 2006), using majority assignment (see Burban et al. (2016) for details). The field data set was then analyzed using parental simulated data as parental reference for INT, and jointly with the simulated data set with NH, to avoid biases linked to unbalanced category sizes. Ten replicates of each NH analysis were performed during 100 000 sweep once the burn-in step was stabilized, using Jeffreys prior for allele frequencies and mixing proportions, to test the consistency of the results. The field individuals for which the h-index fell outside the range of parental simulated data were then considered as hybrid, while the comparison with the type and rate of mis-assignments obtained for the simulated data set was taken into account for NH.

Results

Sampling: distribution of SP and WP individuals over time and space

Although the traps were installed in mid-April in most localities, no male was caught before the 30th of July outside of the Leiria region, except for one individual collected on July 16th in Fundão. Most males were actually trapped between July 30th and September 24th (October 8th in the southernmost site of Tavira), which fully corresponds to the expected period of WP adult activity. In contrast, individuals were caught during both the SP and the WP expected flight seasons in the coastal and in the inner transect, both in 2012 and 2014. Strong variation was nonetheless observed between sites and dates. In particular, the number of trapped SP males was higher than that of WP males in the coastal transect both in 2012 and 2014 (450 SP vs. 21 WP in 2012; 157 SP vs. 59 WP in 2014 over all sites of the coastal transect), while the opposite was true in the inner transect (44 SP vs. 62 WP in 2012 and 55 SP vs. 126 WP in 2014). Variation within transect was also observed, as most of the SP males trapped in the inner transect

were located in the central sites of Monte Bois, Alpedriz and Ataija Cima; and most of the WP males caught in the coastal transect occurred in the northernmost and southernmost sites, i.e., at the limits of the SP range. On the contrary, SP largely outnumbered WP along the coast from Pedrógão to São Martinho. The proportion of SP and WP individuals trapped on both transects in 2012 and 2014 are shown on Figure S1, Supporting Information. Note that after molecular analysis, some of the genotyped WP caught on the coastal transect proved to be LateSP (see Results below), and so the actual number of WP is even lower.

Population genetic analyses

i) Whole data set

This dataset consisted in the 6 populations sampled outside of the Leiria region, as well as the SP and WP individuals caught in both transects in 2012 and 2014. All the analyses we performed showed that eight phenotypically WP individuals (i.e., trapped after July 25th) from the Leiria region were actually assigned to the SP genetic cluster and should thus rather be considered as LateSP individuals sensu Burban et al. (2016). These individuals were thus removed from the WP samples in all population-based analyses such as diversity indices, HWE, LD and pairwise *Fst*. Only few cases of departure from HWE were observed in more than one site (namely *Ppit30* and *Ppit38* in Leiria WP and SP for both years and *Ppit46* in Varges and Viseu). The percentage of null alleles was over 5% only for *Ppit33* and *Ppit30* in 6 and 5 sites, respectively. No pair of loci was consistently in linkage disequilibrium for a majority of sampling sites. Observed (and expected) heterozygosities ranged from 0.43 (0.45) in the SP both in 2012 and 2014 to 0.64 (068 and 0.70) in Viseu and Fundão (Table 1). The two first axes of the PCA performed for the whole dataset is presented in Figure 2, and explained 5.6% and 3.3% of the total variance, respectively. Axis one (PC1) mainly separated the SP from all other populations. Eight WP individuals clearly clustered within the SP group. These individuals will be characterized below, and were consistently identified as belonging to the SP genetic cluster by all subsequent analyses. They are hereafter referred to as LateSP individuals. All winter populations from Portugal are distributed along axis two (PC2) without any clear geographic pattern, as the southern site of Tavira was very close to the northern site of Varges, the three sites of Grândola, Fundão and Viseu overlapped, and Leiria WP and Caparica clustered together (Fig. 2). Axis

three (PC3, not show) explained 2.0% of the total variance and corresponded to a north-south gradient.

Table 1 - Number of genotyped individuals (*N*), number of alleles (*Na*), observed (*Ho*) and expected (*He*) heterozygosities, after removing the LateSP individuals from the data set.

Populations	*N*	*Na*	*Ho*	*He*
Varges	26	150	0.63	0.67
Viseu	24	140	0.64	0.68
Fundão	30	155	0.64	0.70
Caparica	29	91	0.47	0.51
Grândola	30	137	0.61	0.63
Tavira	30	139	0.57	0.64
Leiria WP12	45	128	0.60	0.58
Leiria SP12	100	103	0.43	0.45
Leiria WP14	80	142	0.54	0.58
Leiria SP14	112	106	0.43	0.45

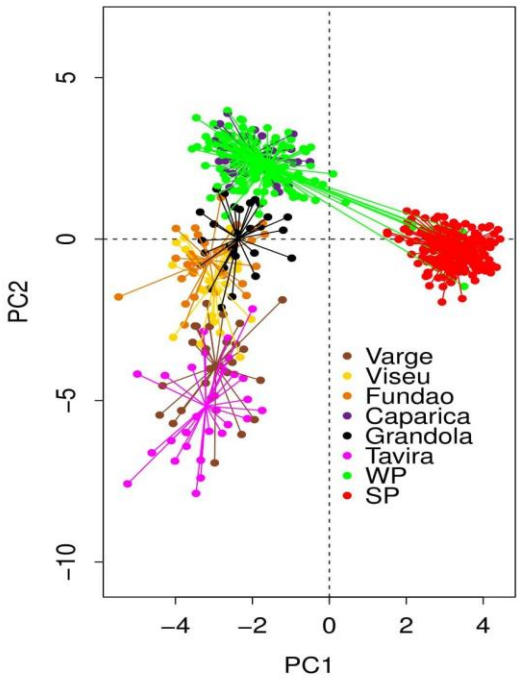

Figure 2 - Graph of the first two axes from principal component analysis (PCA) of microsatellite genotypes of all sampled populations.

The results obtained from STRUCTURE (STR) were consistent with those obtained from PCA. The Log P(X|K) curve reached a plateau for K=6, but ΔK method clearly indicated a highest posterior probability for K=2 (Fig. S2, Supporting information). For K=2, one cluster corresponded to all WP populations while the other corresponded to all SP individuals (Fig. 3) as well as the eight individuals from Leiria WP already identified in the PCA as LateSP (Fig. 2).

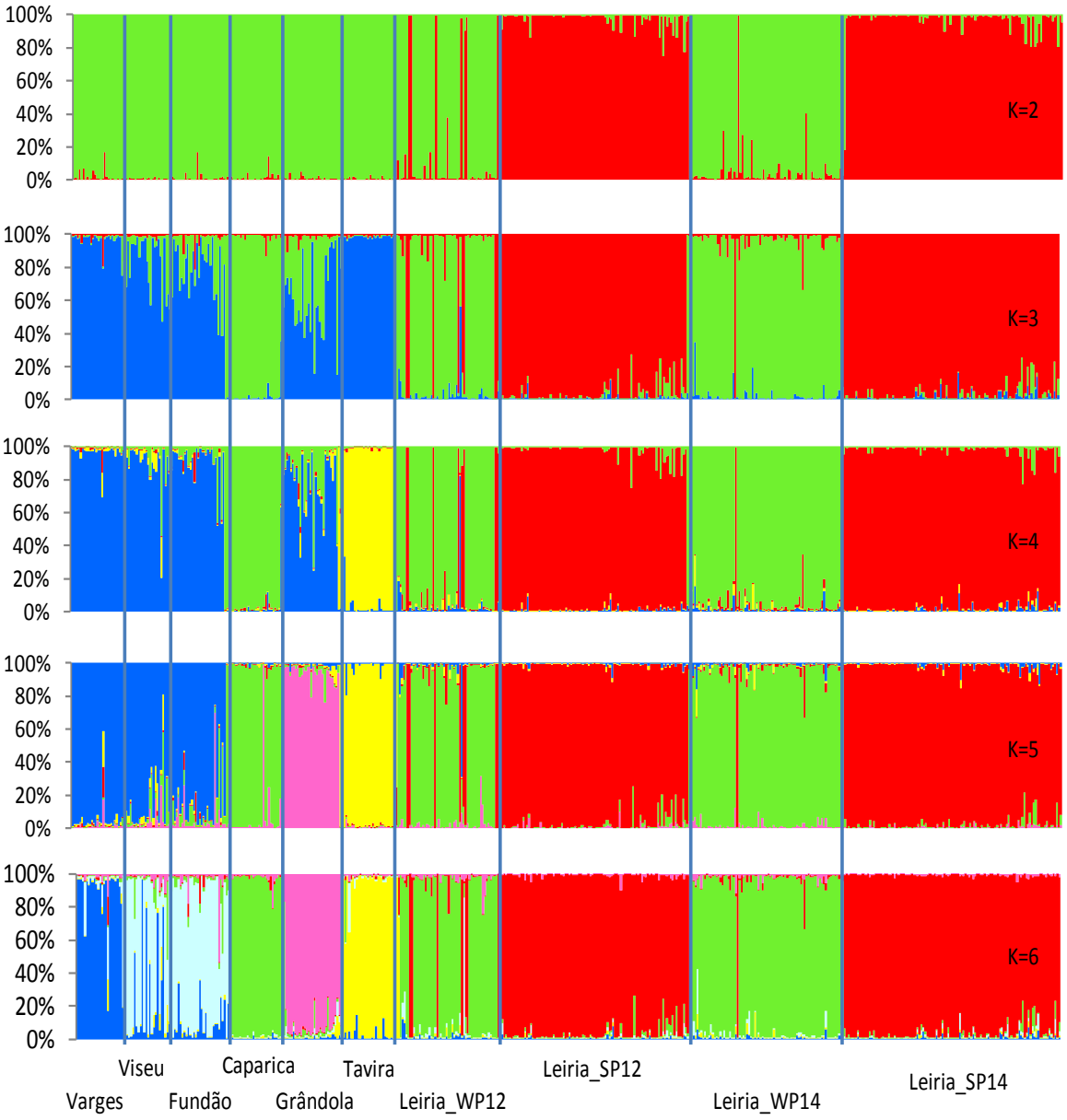

Figure 3 - Graphic representation of the individual genetic assignments given by the individual Q-matrix inferred by STRUCTURE for the 10 populations studied. Individual assignments for K=2 to 6.

These individuals *a priori* identified as WP based on their phenology were actually assigned to the SP genetic cluster with a high Q-value (>0.88) (Table 3). SP individuals were grouped in a specific cluster whatever the value of K. Increasing K led to differentiate some of the WPs. For K=3, Leiria WP and Caparica were grouped in the same cluster, while Tavira and Varges individuals were grouped in the 3rd cluster with high individual Q-values. Individuals from Grândola and to a lesser extent from Viseu and Fundão, were assigned to both of these WP clusters (Fig. 3). For K=4, Tavira formed a separate cluster, while the new clusters obtained for K=5 and K=6 corresponded to Grândola and Varges, respectively. For upper K, results were inconsistent between runs, and individuals exhibited mixed assignments.

Pairwise *Fst* estimated directly or taking into account the excluding null alleles (ENA) correction indicated that Leiria SP (both years) was highly differentiated (0.21 < *Fst* < 0.28) from the other populations characterized by the classical winter phenology, including the sympatric Leiria WP (Table 2).

Table 2 - Pairwise *Fst* between populations, after removing LateSP individuals from the data set. Significant values are presented in bold for a 95% Confidence Interval.

Populations	*Fst*								
	Var	Vis	Fun	Cap	Grâ	Tav	WP 12	SP 12	WP 14
Varges	-								
Viseu	0.04	-							
Fundão	0.05	0.01	-						
Caparica	**0.21**	**0.10**	**0.10**	-					
Grândola	**0.11**	0.06	0.07	**0.08**	-				
Tavira	**0.10**	**0.09**	**0.10**	**0.18**	**0.10**	-			
Leiria WP12	**0.16**	0.07	0.06	0.04	**0.08**	**0.16**	-		
Leiria SP12	**0.26**	**0.24**	**0.25**	**0.27**	**0.22**	**0.28**	**0.24**	-	
Leiria WP14	**0.18**	0.07	0.07	0.03	**0.08**	**0.16**	0.01	**0.21**	-
LeiriaSP14	**0.26**	**0.24**	**0.24**	**0.26**	**0.22**	**0.27**	**0.23**	0.01	**0.21**

Pairwise comparisons between Portuguese winter populations showed that the highest differentiation was found between Caparica and Varges (*Fst* = 0.21) and Caparica and Tavira (0.18). All other pairwise comparisons were comprised between 0.04 and 0.10, except for the population pair Viseu / Fundão that showed a very low *Fst* (0.01). A significant pattern of isolation by distance was found over Portugal (Fig. 4). Finally, no differentiation due to the year of sampling was found neither in the SP nor in the Leiria WP, as *Fst* between SP2012 and SP2014, and between WP2012 and WP2014 was low (0.01) and not significant.

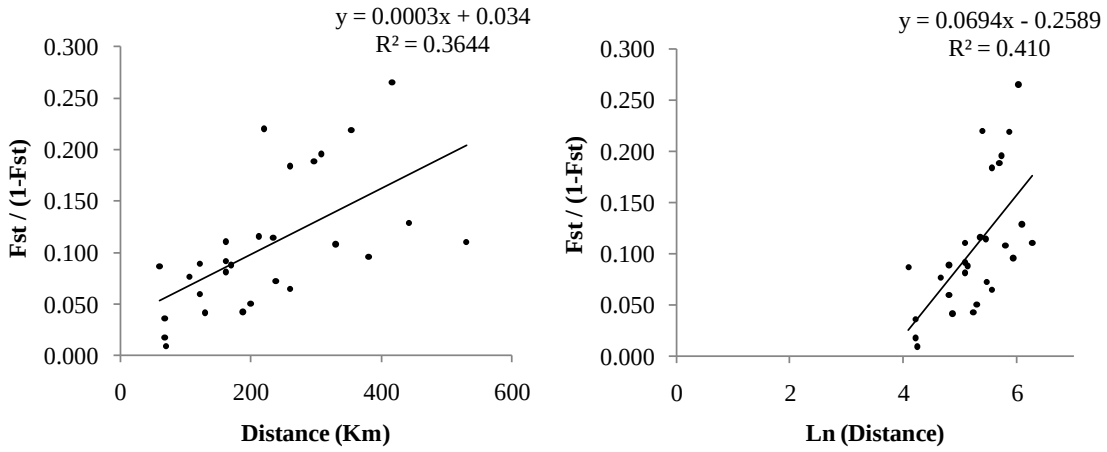

Figure 4 – Isolation by distance between pairs of populations over Portugal, tested by the regression between *Fst* / (1-*Fst*) and either the linear geographic distance either the logarithm of geographic distance.

ii) Coastal and inner transects

A PCA was performed using the SP and the WP individuals trapped in 2012 and 2014 along the two transects. Consistently with previous analyses, the results (not shown) revealed a string differentiation of SP and WP individuals long PC1, and further identified the 8 LateSP individuals. When performed separately within SP and within Leiria WP individuals, the PCA did not revealed any structuration within population (Fig. S3, Supporting information). In the same way, when STRUCTURE analysis was performed including all individuals from the transects, it clearly separated a SP from a WP genetic cluster, with the exception of the 8 LateSP. When run separately on Leiria SP and Leiria WP excluding these LateSP individuals, STRUCTURE results unambiguously showed that K=1 was the best solution (not shown).

We also calculated pairwise *Fst* between years and sampling sites along the two transects excluding sites where sampling size was lower than 10 individuals. Consistently with other results, pairwise *Fst* between Leiria SP and WP samples from any site and year remained high ($0.16 < Fst < 0.29$), while the differentiation between sites within the SP or within the WP was consistently low, reaching 0.03 at most (Table S3, Table S4, Supporting information). No pattern of isolation by distance was found.
Characteristics of the 8 LateSP individuals are given in Table 3. They were all identified in the coastal transect, mostly in sites where "true" WP (i.e., identified as such both from their phenotype and genetic clustering) were absent (Pedrogão, Praia Vieira, T171) or very rare (Nazaré), with the notable exception of one LateSP individual identified as far south as Cabeça Gorda, outside of the known SP range (Fig. 6). Consistently with previous results (Burban et al. 2016), 3 LateSP were caught in the beginning of the WP flight season (end of July to early August), while the other 5 were trapped as late as September.

Table 3 – Characterization of the eight LateSP individuals: individual code, geographic location, date of capture and Q-value for the inferred clusters given by STRUCTURE for the whole data set.

Individual code	Locality	Date of capture	Q-value
WP12-008	Pedrógão	27/07/2012	0.997
WP12-002	Nazaré	06/09/2012	0.986
WP12-004	Nazaré	06/09/2012	0.881
WP12-005	Nazaré	06/09/2012	0.986
WP12-007	Praia Vieira	06/09/2012	0.997
WP12-006	T171	29/09/2012	0.997
N2471	Nazaré	24/07/2014	0.996
C782	Cabeça Gorda	07/08/2014	0.954

Hybrid detection

Analyses of the simulated data with INT revealed that h-index ranged from 0 to 0.243 for pSPsim and from 0.642 to 1 for pWPsim. The ranges of h-index obtained for simulated hybrid categories greatly overlapped with each other and therefore could not be used to discriminate between hybrid categories (not shown). INT identified 7 field

individuals as hybrids, i.e., their h-index values fell outside the ranges obtained for simulated parents. These hybrid individuals corresponded to 1 WP trapped in São Martinho do Porto in 2014, showing a h-index value of 6.640 close to the h-index obtained for pWPsim and 6 SP (trapped in Nazaré and Monte Bois in 2012, São Martinho do Porto in 2012 and 2014, and São Gregório in 2014) with h-index values ranging from 0.244 to 0.303, close to the h-index obtained for pSPsim (Fig. 5).

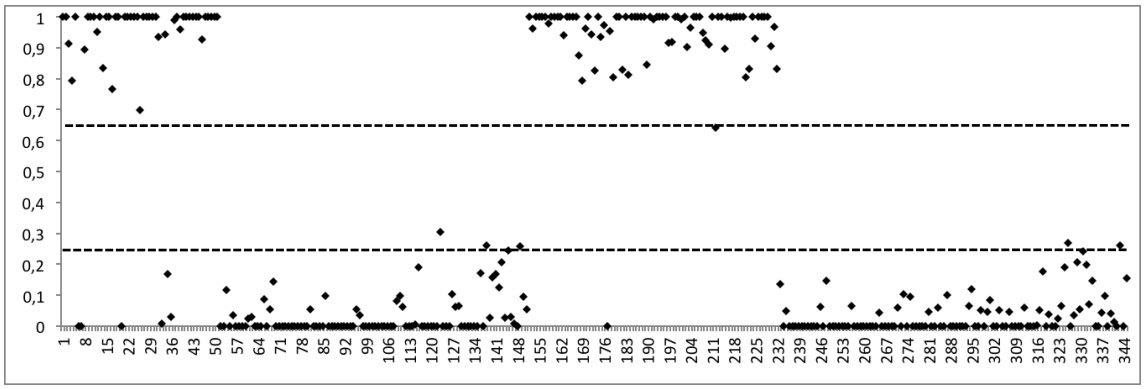

Figure 5 – h-index of field individuals (1-51: WP2012; 52-151: SP2012; 152-233: WP2014; 234-345: SP2014) calculated with parental simulated data as reference (0 = pure SP, 1 = pure WP). Dashed lines represent the limit values of h-index obtained for the simulated parental genotypes.

Performances in hybrid detection with NH are summarized in Table 4. Both efficiency and accuracy were high for parental categories while they were lower for hybrids. Parental individuals could however be mis-assigned in their respective back-cross categories, and backcross individuals to their respective parental category (Table 5). More, as some simulated parents were mis-assigned as backcrosses and exhibited a posterior probability of 1 to be in a hybrid category, no threshold could be defined to ascertain any backcross identity. Therefore, it would have been possible to ascertain the hybrid origin only for individual assigned as F1 and F2, but no such individual was identified using NH among the samples trapped in the transects.

Table 4 - Performances of NH in detecting genetic class using majority assignment evaluated with 1000 simulated genotypes of each class.

	Efficiency	Accurency	Overall Performance
SP	0.942	0.935	0.881
WP	0.877	0.871	0.764
F1	0.822	0.745	0.613
F2	0.535	0.724	0.387
bcSP	0.794	0.751	0.596
bcWP	0.721	0.663	0.478

However, while the percentage of field individuals assigned as bcWP was similar to the percentage of pWPsim mis-assigned as bcWP (12%), the proportion of field individuals assigned as bcSP (13.2%) was much higher than the proportion of pSPsim mis-assigned in this category (5.8%). To sum up, most individuals (except the LateSP) exhibited h-index and NH assignment relevant to their phenotypical parental categories.

Table 5 - Proportion of simulated genotypes assigned to each genetic class with NH.

	pSP	pWP	F1	F2	bcSP	bcWP
pSPsim	0.942	0.000	0.000	0.000	0.058	0.000
pWPsim	0.000	0.877	0.000	0.003	0.000	0.120
F1sim	0.000	0.000	0.822	0.053	0.075	0.050
F2sim	0.002	0.005	0.135	0.535	0.158	0.165
bcSPsim	0.063	0.000	0.077	0.065	0.794	0.001
bcWPsim	0.000	0.125	0.069	0.083	0.002	0.721

All the 7 individuals identified as hybrid from INT analysis were all assigned as back-cross with NH (6 bcSP and 1 bcWP, which is consistent with their h-index values) (Table 6). All identified hybrids had a phenology consistent with their type of backcross. They were all located in the southern part the SP range (Fig. 6).

Table 6 - Characteristics of the hybrids detected by their h index.

Name	h index	NH maj. ass.	Date	Location
SP12-190	0.303	bcSP 0.670	29/06/2012	Nazaré
SP12-238	0.259	bcSP 0.848	29/06/2012	Monte Bois
SP12-181	0.244	bcSP 0.892	29/06/2012	S. Martinho
SP12-187	0.256	bcSP 0.643	29/06/2012	S. Martinho
WP14-S781	0.640	bcWP 0.557	07/08/2014	S. Martinho
SP14-S1268	0.269	bcSP 0.599	12/06/2014	S. Martinho
SP14-G1263	0.259	bcSP 0.725	12/06/2014	S. Gregório

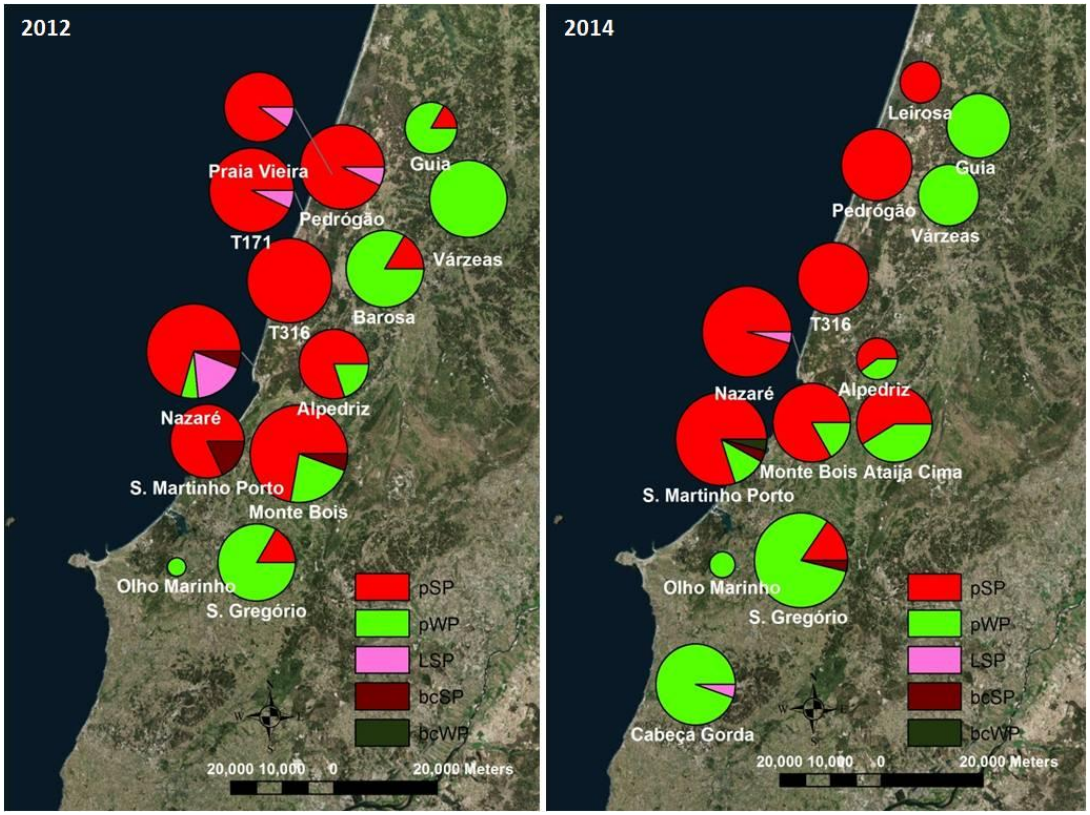

Figure 6 - Genetic assignments of 345 males trapped in the two transects (Coastal and Inner) of Leiria region in 2012 and 2014. Only hybrids detected from both Introgress and NH analysis appeared in hybrid categories.

Discussion

Genetic structure at different spatial scales and differentiation of the SP

The present study showed a general pattern of isolation by distance for the WPs sampled over Portugal. Such a result was expected for this insect species, as IbD was previously evidenced in France over similar geographic distances when physical geographical barriers (namely the Massif Central) were taken into account (Kerdelhué et al., 2006). Groups of sampled sites with very low pairwise differentiation indices nonetheless show that gene flow is favored in some regions, suggesting a high connectivity of populations in these areas (e.g., *Fst* values below 0.05 between Varges, Viseu and Fundão, i.e. the north-eastern sites; and between Caparica and Leiria WP, i.e., along the coast in the central part of Portugal). On the other hand, high *Fst* values (reaching 0.16 - 0.21) were obtained between the Leiria WP + Caparica group and the two most distant sites of Varges in the North and Tavira in the South of Portugal, which suggests that barriers to gene flow impedes PPM migration between these locations. Such a pattern of strong vs. weak genetic structure in particular regions could be due to some landscape features resulting in ecological discontinuities while others favor connectivity of moth populations. Indeed, the characteristics of land use over Portugal show a heterogeneous distribution. In particular, *Eucalyptus* plantations are mainly present in Central Litoral combined with some agricultural areas, whereas evergreen oak forests, cork oak and holm oak, dominate in Southern Portugal. These land uses probably limit PPM dispersal while pine and mixed forests could favor its propagation. Moreover, open habitats and agricultural lands, where isolated pine trees were proved to help stepping stone moth movements (Rossi et al., 2016), can also bridge otherwise distant PPM populations. Whether landscape features can explain to some extent the spatial patterns found here could be specifically addressed in the future using an appropriate sampling design and detailed landscape descriptors.

The indices of differentiation between the SP and any of the sampled WPs were systematically higher than those estimated between WPs. They were comprised between 0.21 and 0.28, which was consistent with previous results based on lower numbers of sampling sites and genetic markers (Santos et al., 2007; 2011a; Burban et al., 2016). This result confirms that the SP is strongly differentiated from all regional PPM populations with typical phenology, possibly due to bottleneck events and a strong genetic drift. The fact that the *Fst* values remain high between the 2 allochronic

populations in the most recent sampling campaigns of 2012 and 2014 suggests that the rare hybridization events identified by Burban et al. (2016) do not tend to decrease differentiation. Interestingly, the Fst values obtained between the SP and any WP was lowest when considering the sympatric Leiria WP and highest when choosing the southernmost Tavira population. Note that the genetic characteristics of the SP were very stable in space and time, as no differentiation was evidenced neither between sampling years nor among the sites sampled within the SP distribution range (Table S3, Table S4, Supporting information). No pattern of isolation by distance was found within both transects.

Variability and overlap of SP and WP flight periods

The SP was first discovered in 1997 in the southern MNL and has been monitored since then using pheromone traps, mostly in its core area. A striking result highlighted in previous works (Pimentel et al., 2006; Santos et al., 2007; 2011a) was that the SP males were trapped until early July each year while the emergence of WP started in late July, which resulted in a 2 to 4 weeks gap during which no male were caught in the field. The only exception was a male collected in mid-July in 2009, which was later proved to be a F1 hybrid and to exhibit an intermediate phenology (Burban et al., 2016), consistently with experimental crosses (Branco et al., 2016). Using genetic assignments of a small fraction of the males trapped between 2007 and 2010, we further discovered that few individuals trapped at the beginning (late July) or the end (September) of the WP flight period turned out to belong to the SP and were described as "LateSP" individuals. Interestingly, such atypical SP males were all trapped in the SP core area (southern area of the MNL). Monitoring data of the corresponding years (2007 and 2010) still exhibited in July the expected gap between the emergence curves.

The systematic sampling we used in 2012 and 2014 for the present study encompassed most of the SP distribution range as delimited by Godefroid et al. (2016) from a thorough field survey, and thus included sites with more diversified environmental conditions than previous studies. Moreover, we genetically assigned males trapped in each sampling site of both transects for each date (except in few cases when samples could not be properly conserved) over the whole adult emergence period, which was not the case in the past. This procedure showed that a few males were trapped during the expected gap between SP and WP flight curves both in 2012 and 2014, and were

identified as "pure" SP or WP individuals rather than belonging to any hybrid category. For instance, one SP male was trapped in the north (Pedrogão) while one WP male was caught in the south (São Gregório) during the second half of July 2012; similarly, 3 WP (Cabeça Gorda and São Gregório) and 1 SP (Nazaré) males were trapped in the same period in 2014. It is worth noting that the two above-mentioned SP males were both trapped in sites where no WP individuals were subsequently observed. One SP individual was collected in the beginning of August in the southernmost location of Cabeça Gorda (Table S2) where only WP individuals were otherwise found and no SP nest was observed so far. Moreover, as in Santos et al. (2011a) and Burban et al. (2016), we identified 5 SP individuals emerging in 2012 at the very end of summer. Taken together, these observations show that dates of emergence of SP males are actually highly variable, and can overlap with the WP flight period, even if a large majority (99%) of SP males do emerge before mid-July. The sampling design developed for the present study, encompassing the whole SP spatial and temporal range, was adequate to reveal the phenotypic variability of this peculiar population and to go beyond previous results. Micro-environmental conditions can be involved in the delayed phenology of some SP males (especially for those emerging in the middle of summer, corresponding to "moderately LateSP"), but an abnormal process of diapause or diapause termination could occur in the very delayed SP males that are trapped in September. Both types of LateSP can occur in a single site (e.g., in Nazaré 3 very delayed LateSP were found in 2012 and a moderately LateSP male was trapped in 2014). They were trapped all over the coastal transect, from Cabeça Gorda to Pedrogão, and were thus not restricted to the MNL as suggested by the results shown by Burban et al. (2016). If the very delayed LateSP could be counter-selected because of their very late emergence, the few individuals identified as "moderately Late SP" trapped at the end of July probably correspond to the very end of the SP flight period, which should be seen as a curve with a long, flat tail-end rather than a normal distribution, and can in some cases overlap with the beginning of the WP flight. Even if this phenomenon concerns only few individuals some years, it could lead to hybridization events between the two allochronic populations. As opposed to the SP males, WP individuals were consistently trapped between late July and late September, suggesting that all individuals caught from May to early July can be confidently identified as SP individuals. The monitoring results nonetheless showed some inter-annual variations probably due to local climatic conditions that are known to modulate the phenology of adult emergence in the PPM

(Battisti et al., 2006). For instance, the first male was trapped on May 4th in 2012 while the first catch was recorded on May 14th in 2014. To complete the picture, one can mention that, consistent with the observations of Burban et al. (2016), the individuals identified as back-crosses emerged within the flight season of their corresponding parental category. They were all found in the southern part of the SP range, from Nazaré to São Gregório.

Fine-scale spatial distributions of both populations in the SP range

When the SP was first discovered in the southern part of the MNL in 1997, the WP was present throughout the region although at low densities. Pimentel et al. (2006) mentioned the even distribution of WP in all the plots surveyed within the MNL. Trees defoliated the same year by both populations successively were observed (H. Santos, pers. comm.). Since 1997, the SP has extended geographically along the coast mostly southwards, and northwards in a lesser extent. Species distribution modeling approaches suggested that climate would be the main factor preventing its propagation further East (Godefroid et al., 2016). The results presented here show that the WP occurred in the northern, southern and eastern parts of the monitored region, whereas its absence or scarcity in the core coastal locations was remarkable. In the 2007-2010 field surveys, the WP was absent in the extreme south of the MNL, where the first SP outbreak was observed in 1997, and in Nazaré; the two populations were nonetheless found in sympatry at the northern and southern limit of the SP range, respectively São Martinho and north of the MNL (Burban et al., 2016). The 2012-2014 data set presented here evidenced that the two populations do co-occur along the inland transect, but that WP is now absent or scarce in sites where it used to be found in sympatry with the SP few years ago (north and south of the coastal transect) even if WP already exhibited low population sizes. Specific studies will be needed to determine whether this tendency will continue in the future. If the apparent displacement of the WP from the coastal transect can be proven, the factors explaining this trend, such as a process of competitive exclusion, will have to be elucidated.

Yet, even if the main co-existence pattern suggests a decrease of the WP along the coast, some inter-annual variations were observed and contradicted this view. In particular, even if no WP males were recorded in Nazaré from 2007 to 2010 and in 2014, one was trapped in 2012 in the same site. Similarly, WP males were found in São

Martinho in 2014, even though none were trapped in 2012. It could be hypothesized that WP could re-colonize areas from which it disappeared in the previous years. On the other hand, such records could correspond to isolated long-distance dispersal events from surrounding stands as PPM males have good flying capacities and can be efficiently attracted by the pheromone lures used here (Salvato et al., 2005). To discriminate between these two hypotheses, and to infer in detail the fine-scale co-occurrence patterns of the two populations, monitoring and quantifying SP and WP nests along the two transects will be necessary.

The data presented here and the experience accumulated on the study system suggest that the differentiating populations were once truly in sympatry within the Mata Nacional de Leiria, and that the system now moved toward a fine-scale parapatric pattern. Density-dependent processes could be at play, and dedicated studies should now be developed to study the indirect interactions between the allochronic populations. Based on the knowledge gained so far, we could represent the differentiation scenario of the SP and the local WP as the Dieckman's cube (Dieckman et al., 2004) shown on Figure 7. We currently hypothesize that (i) a strong shift in phenology is the primary cause of mating disruption due to the mostly-non overlapping reproductive seasons. As a result, the differentiating SP experienced radically different environmental conditions and (ii) started to adapt (e.g., proven increased tolerance of the larvae to high summer temperatures (Santos et al., 2011b); changes in reproductive traits and reproductive success (Santos et al., 2013; Rocha et al., 2017), leading to further adaptive differentiation between populations. The current step of this on-going differentiation scenario (this study) suggests that (iii) the system is currently tending to move from strict sympatry to fine-scale parapatry, which further impedes gene flow and introgression between populations, which could explain the scarcity of hybrids identified in the field. Yet, speciation is still not an achieved process. Future climate changes could modulate further ecological and spatial differentiation, as well as phenology, the main factor implicated so far in reproductive isolation.

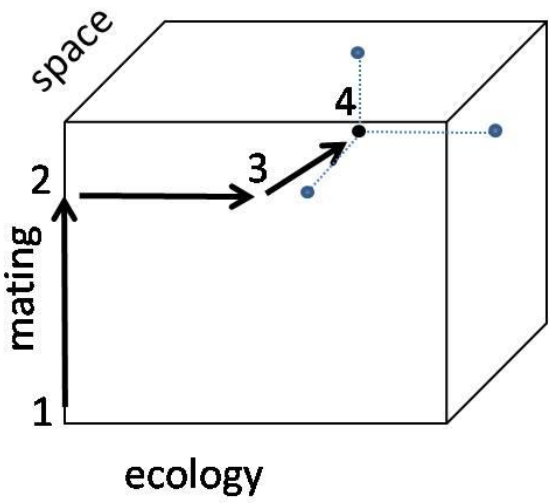

Figure 7 - Chronology of SP differentiation during ongoing speciation from WP regarding ecology, mate choice and geographic distribution, represented on a Dieckman's cube. 1-2: From the WP, a primary mutationnal step in the Leiria zone was responsable for allochronic differentiation of the SP. 2-3: The shift of phenology submits the SP to new environmental conditions during its development, causing divergent selection. 3-4: Environmental characteristics delimit the expansion area of the SP, while competition excludes the WP in the core area of the SP.

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Supporting information

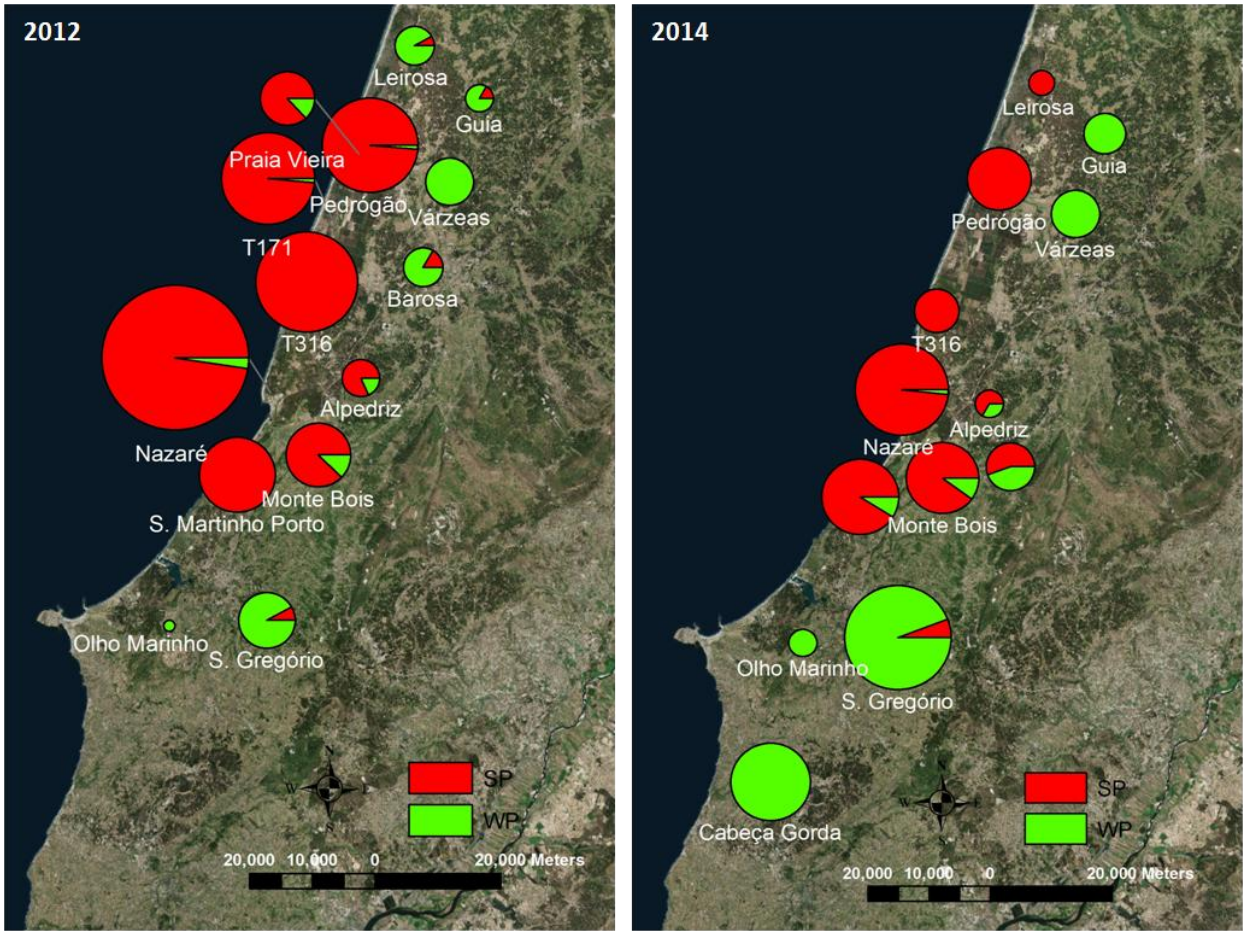

Figure S1 – Number of SP and WP males trapped on both transects in 2012 and 2014.

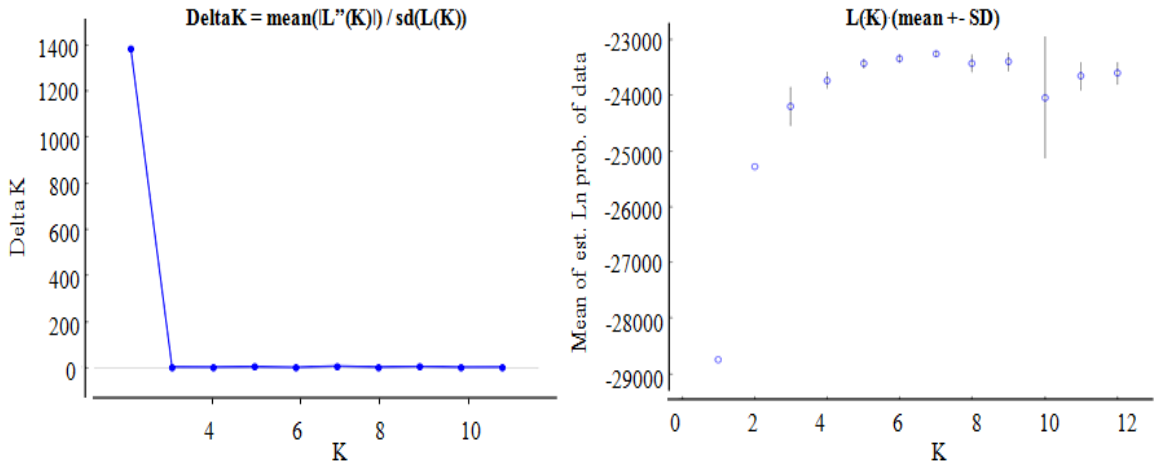

Figure S2 – Curve of ΔK obtained with the Evanno et al (2005) method (left) and curve of LnP(X|K) as a function of K (right) corresponding to structure results obtained on the whole data set with K varying from 1 to 12.

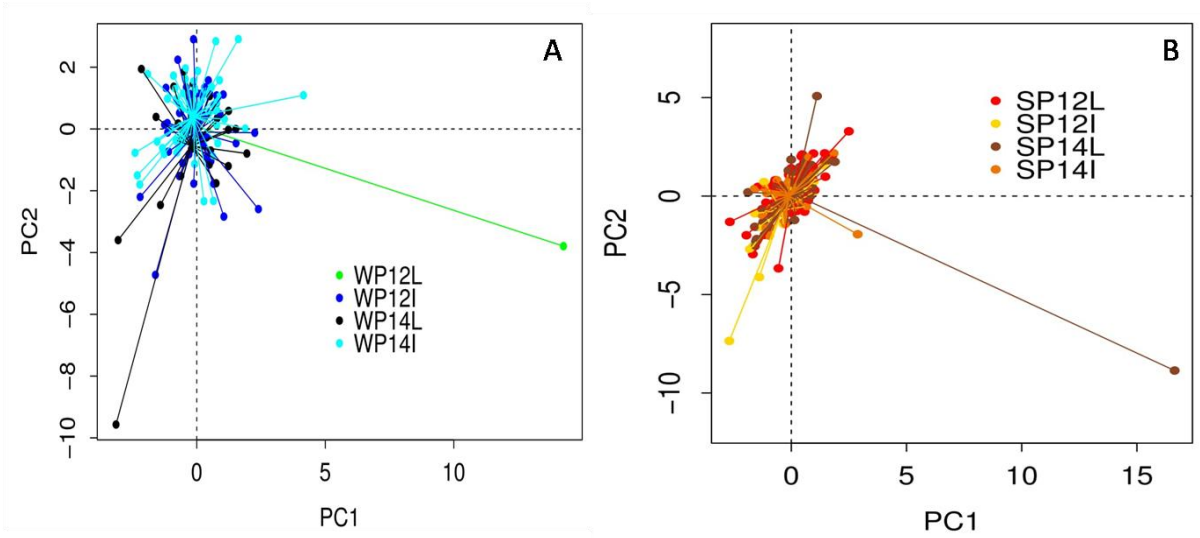

Figure S3 - Graphs of the first two axes from principal component analysis (PCA) of microsatellite genotypes of the: A - Leiria WP excluding LateSP individuals and B – Leiria SP.

Table S1 - Sampled localities and number of captured and genotyped individuals of the *Thaumetopoea pityocampa* populations. SP: Summer Population phenology, WP: Winter Population phenology.

	Populations /locality	**Geographical coordinates**	**Transect**	**Phenology**	**Year of collection**	**Captured males**	**Genotyped individuals**
	Varges	N41º52'16.6'' W6º40'44.0''	-	WP	2012	NA	26
	Viseu	N40º34'16.78"; W8º0.5'28.33"	-	WP	2012	NA	24
	Fundão	N40º09'08.21" W7º30'59.31"	-	WP	2012	194	30
	Caparica	N38º39'49.76" W9º12'38.47"	-	WP	2012	42	29
	Grândola	N38º15'05.4" W8º45'32.6"	-	WP	2012	50	30
	Tavira	N37º11'03.16" W7º34'27.62"	-	WP	2012	56	30
Leiria region	Leirosa	N40º00'04.20" W8º51'54.13"	Coastal	WP	2012 2014	11* 0	0 0
				SP	2012 2014	1* 5	0 5
	Pedrógão	N39º53'26.4" W8º55'47.8"	Coastal	WP	2012 2014	1 0	1 0
				SP	2012 2014	71 31	13 15
	Praia Vieira	N39º52'50.3" W8º57'15.2"	Coastal	WP	2012 2014	3 0	1 0
				SP	2012 2014	20 0	9 0
	T171 (MNL)	N39º47'50.45" W8º58'47.0"	Coastal	WP	2012 2014	1 not sampled**	1
				SP	2012 2014	67 not sampled**	13
	T316 (MNL)	N39º44'17.0" W9º1'20.8"	Coastal	WP	2012 2014	0 0	0 0
				SP	2012 2014	81 15	14 15
	Nazaré	N39º36'57.05" W9º4'28.14"	Coastal	WP	2012 2014	4 1	4 1
				SP	2012 2014	165 65	13 23
	S. Martinho Porto	N39º31'19.8" W9º7'18.7"	Coastal	WP	2012 2014	0 4	0 4
				SP	2012 2014	45 41	11 21
	Olho Marinho	N39º21'9.5" W9º13'8.1"	Coastal	WP	2012 2014	1 6	1 2
				SP	2012 2014	0 0	0 0

Leiria region	Cabeça Gorda	N39º11'26.71" W9º15'58.93"	Coastal	WP	2012	not sampled*	
					2014	48	19
				SP	2012	not sampled*	
					2014	0	0
	Guia	N39º56'34.2" W8º46'31.3"	Inner	WP	2012	5	5
					2014	13	12
				SP	2012	1	1
					2014	0	0
	Várzeas	N39º51'0.1" W8º49'5.8"	Inner	WP	2012	18	12
					2014	18	11
				SP	2012	0	0
					2014	0	0
	Barosa	N39º45'14.8" W8º51'21.6"	Inner	WP	2012	10	10
					2014	not sampled*	
				SP	2012	2	2
					2014	not sampled*	
	Alpedriz	N39º37'48.9" W8º56'41.4"	Inner	WP	2012	2	2
					2014	2	2
				SP	2012	9	8
					2014	4	3
	Ataija Cima	N39º33'26.6" W8º54'50.13"	Inner	WP	2012	0	0
					2014	8	7
				SP	2012	0	0
					2014	10	10
	Monte Bois	N39º32'40.7" W9º0'15.85"	Inner	WP	2012	4	4
					2014	4	3
				SP	2012	29	14
					2014	36	15
	S. Gregório	N39º21'32.8" W9º4'47.2"	Inner	WP	2012	23	10
					2014	81	21
				SP	2012	2	2
					2014	5	5

* Samples lost due to predator attacks in the traps
** Places not sampled in the referred year due to logistic impediments.

Table S2 - Number of males trapped and genotyped in the two transects by locality and by date of collect, in 2012 and 2014. Colors indicate the results of genetic assignments (red: pSP, pink: Late pSP, green: pWP, yellow: bcSP, brown bcWP).

2012		May 4	May 17	May 31	Jun 14	Jun 29	Jul 13	Jul 27	Aug 8	Aug 22	Sep 6	Sep 20
Olho Marinho	Trapped	0	0	0	0	0	0	0	0	1	0	0
	Genotyped	0	0	0	0	0	0	0	0	1	0	0
S. Martinho	Trapped	1	3	9	21	10	1	0	0	0	0	0
	Genotyped	1	1	1	3	2 - 2	1	0	0	0	0	0
Nazaré	Trapped	1	8	46	108	2	0	0	0	0	4	0
	Genotyped	1	1	4	6	1	0	0	0	0	1 - 3	0
T316	Trapped	0	0	19	36	25	1	0	0	0	0	0
	Genotyped	0	0	6	4	3	1	0	0	0	0	0
T171	Trapped	0	2	12	34	19	0	0	0	0	0	1
	Genotyped	0	0	5	5	3	0	0	0	0	0	1
Praia Vieira	Trapped	0	0	0	17	3	0	0	1	0	1	1
	Genotyped	0	0	0	7	2	0	0	0	0	1	0
Pedrógão	Trapped	3	5	26	33	3	1	1	0	0	0	0
	Genotyped	0	0	6	4	2	1	1	0	0	0	0
Leirosa	Trapped	0	0	1	0	0	0	0	11	0	0	0
	Genotyped	0	0	0	0	0	0	0	0	0	0	0
Guia	Trapped	0	0	0	1	0	0	0	0	5	0	0
	Genotyped	0	0	0	1	0	0	0	0	5	0	0
Várzeas	Trapped	0	0	0	0	0	0	0	11	7	0	0
	Genotyped	0	0	0	0	0	0	0	8	4	0	0
Barosa	Trapped	0	0	0	1	1	0	0	5	5	0	0
	Genotyped	0	0	0	1	1	0	0	5	5	0	0
Alpedriz	Trapped	0	1	0	6	2	0	0	0	2	0	0
	Genotyped	0	1	0	6	1	0	0	0	2	0	0
Monte Bois	Trapped	0	0	6	21	2	0	0	0	3	1	0
	Genotyped	0	0	6	6	1 - 1	0	0	0	3	1	0
Ataija Cima	Trapped	0	0	0	0	0	0	0	0	0	0	0
	Genotyped	0	0	0	0	0	0	0	0	0	0	0
S. Gregório	Trapped	0	0	0	1	1	0	1	5	8	6	3
	Genotyped	0	0	0	1	1	0	1	3	3	1	2

Table S2 (cont.) - Number of males trapped and genotyped in the two transects by locality and by date of collect, in 2012 and 2014. Colors indicate the results of genetic assignments (red: pSP, pink: Late pSP, green: pWP, yellow: bcSP, brown bcWP).

2014		May 1	May 8	May 14	May 23	May 29	Jun 05	Jun 07	Jun 12	Jun 18	Jun 21	Jun 26	Jul 05	Jul 10	Jul 19	Jul 24	Aug 7	Aug 21	Sep 4	Sep 18	Oct 10	Oct 22
Cabeça Gorda	Trapped	0	-	0	-	0	-	-	0	-	-	0	-	0	-	2	22	17	7	0	0	0
	Genotyped	0	-	0	-	0	-	-	0	-	-	0	-	0	-	2	5 - 1	6	5	0	0	0
Olho Marinho	Trapped	0	-	0	-	0	-	-	0	-	-	0	-	0	-	0		0	2	4	0	0
	Genotyped	0	-	0	-	0	-	-	0	-	-	0	-	0	-	0		0	2	0	0	0
S. Martinho	Trapped	0	-	1	-	13	-	-	22	-	-	4	-	1	-	0	3	1	0	0	0	0
	Genotyped	0	-	1	-	7	-	-	1 - 7	-	-	4	-	1	-	0	1 - 2	1	0	0	0	0
Nazaré	Trapped	0	-	4	-	22	10	-	13	9	-	7	-	0	-	1	0	0	0	0	0	0
	Genotyped	0	-	3	-	3	4	-	5	6	-	2	-	0	-	1	0	0	0	0	0	0
T316	Trapped	0	-	0	-	1	-	-	9	-	-	4	-	1	-	0	0	0	0	0	0	0
	Genotyped	0	-	0	-	1	-	-	9	-	-	4	-	1	-	0	0	0	0	0	0	0
Praia Vieira	Trapped	-	0	-	0	-	-	0	-	-	0	-	0	-	0	0	0	0	0	0	0	0
	Genotyped	-	0	-	0	-	-	0	-	-	0	-	0	-	0	0	0	0	0	0	0	0
Pedrógão	Trapped	-	0	-	4	-	-	1	-	-	26	-	0	-	0	0	0	0	0	0	0	0
	Genotyped	-	0	-	4	-	-	1	-	-	10	-	0	-	0	0	0	0	0	0	0	0
Leirosa	Trapped	-	0	-	1	-	-	4	-	-	0	-	0	-	0	0	0	0	0	0	0	0
	Genotyped	-	0	-	1	-	-	4	-	-	0	-	0	-	0	0	0	0	0	0	0	0
Guia	Trapped	-	0	-	0	-	-	0	-	-	0	-	0	-	0	0	3	6	4	0	0	0
	Genotyped	-	0	-	0	-	-	0	-	-	0	-	0	-	0	0	2	6	4	0	0	0
Várzeas	Trapped	0	-	0	-	0	-	-	0	-	-	0	-	0	-	0	0	15	1	2	0	0
	Genotyped	0	-	0	-	0	-	-	0	-	-	0	-	0	-	0	0	8	1	2	0	0
Alpedriz	Trapped	0	-	0	-	0	-	-	1	-	-	3	-	0	-	0	1	1	0	0	0	0
	Genotyped	0	-	0	-	0	-	-	1	-	-	2	-	0	-	0	1	1	0	0	0	0
Monte Bois	Trapped	0	-	1	-	2	-	-	18	-	-	15	-	0	-	0	0	2	1	1	0	0
	Genotyped	0	-	1	-	2	-	-	7	-	-	5	-	0	-	0	0	2	0	1	0	0
Ataija Cima	Trapped	0	-	0	-	3	-	-	1	-	-	6	-	0	-	0	0	5	2	0	1	0
	Genotyped	0	-	0	-	3	-	-	1	-	-	6	-	0	-	0	0	4	2	0	1	0
S. Gregório	Trapped	0	-	0	-	1	-	-	3	-	-	1	-	0	-	1	7	11	37	24	1	0
	Genotyped	0	-	0	-	1	-	-	1 - 2	-	-	1	-	0	-	1	3	4	6	6	1	0

Table S3 – Pairwise *Fst* matrix between sites (with N>10) of the transects data sets obtained from microsatellite data using FREENA with applying the ENA correction for the null alleles. LateSP individuals were excluded from the data. Significant values are presented in bold using the 95% confidence interval.
Site codes: P: Pedrógão; N: Nazaré; SM: São Martinho; AT: Ataija Cima; MB: Monte Bois; V: Várzeas; B: Barosa; SG: São Gregório.

Site	MB	P	T171	T316	N	SM	AT	MB	P	T316	N	SM	V	B	SG	G	V	SG
Population and year	SP12	SP12	SP12	SP12	SP12	SP12	SP14	SP14	SP14	SP14	SP14	SP14	WP12	WP12	WP12	WP14	WP14	WP14
P_SP12	**0.030**																	
T171_SP12	0.015	0.006																
T316_SP12	0.012	0.014	0.002															
N_SP12	**0.035**	0.007	**0.027**	0.015														
SM_SP12	**0.030**	**0.020**	**0.021**	0.014	0.014													
AT_SP14	0.004	**0.028**	0.006	0.015	0.018	0.026												
MB_SP14	**0.022**	0.011	0.013	0.024	0.031	0.025	0.012											
P_SP14	**0.032**	0.008	0.012	0.031	0.018	**0.027**	0.007	0.018										
T316_SP14	0.008	0.008	-0.006	0.007	**0.039**	**0.022**	-0.004	0.011	0.016									
N_SP14	0.018	0.017	0.031	0.022	0.015	**0.024**	0.004	0.015	0.005	0.018								
SM_SP14	0.001	**0.017**	**0.021**	0.022	**0.021**	0.013	0.000	0.008	0.014	0.005	0.006							
V_WP12	**0.177**	**0.224**	**0.218**	**0.201**	**0.206**	**0.154**	**0.183**	**0.202**	**0.207**	**0.218**	**0.196**	**0.167**						
B_WP12	**0.213**	**0.263**	**0.257**	**0.240**	**0.249**	**0.189**	**0.214**	**0.246**	**0.241**	**0.255**	**0.229**	**0.205**	0.001					
SG_WP12	**0.219**	**0.285**	**0.266**	**0.256**	**0.268**	**0.210**	**0.215**	**0.258**	**0.256**	**0.267**	**0.244**	**0.222**	**0.025**	0.017				
G_WP14	**0.200**	**0.252**	**0.241**	**0.231**	**0.237**	**0.175**	**0.197**	**0.231**	**0.252**	**0.238**	**0.237**	**0.205**	**0.026**	0.032	0.041			
V_WP14	**0.206**	**0.261**	**0.252**	**0.241**	**0.248**	**0.197**	**0.207**	**0.248**	**0.240**	**0.251**	**0.232**	**0.209**	0.004	-0.005	-0.004	0.030		
SG_WP14	**0.200**	**0.246**	**0.236**	**0.222**	**0.231**	**0.171**	**0.192**	**0.229**	**0.233**	**0.232**	**0.223**	**0.195**	**0.019**	0.024	0.009	0.011	0.027	
CG_WP14	**0.185**	**0.223**	**0.220**	**0.201**	**0.208**	**0.147**	**0.191**	**0.211**	**0.214**	**0.214**	**0.208**	**0.179**	0.013	0.021	**0.037**	**0.024**	**0.020**	**0.026**

Table S4 – Pairwise *Fst* matrix between sites (with N>10) of the transects data sets obtained from microsatellite data using FREENA without applying the ENA correction for the null alleles. LateSP individuals were excluded from the data. Significant values are presented in bold using the 95% confidence interval.

Site codes: P: Pedrógão; N: Nazaré; SM: São Martinho; AT: Ataija Cima; MB: Monte Bois; V: Várzeas; B: Barosa; SG: São Gregório.

Site Population and year	MB SP12	P SP12	T171 SP12	T316 SP12	N SP12	SM SP12	AT SP14	MB SP14	P SP14	T316 SP14	N SP14	SM SP14	V WP12	B WP12	SG WP12	G WP14	V WP14	SG WP14
P_SP12	**0.031**																	
T171_SP12	0.014	0.004																
T316_SP12	0.009	0.013	-0.002															
N_SP12	**0.031**	0.004	0.022	0.013														
SM_SP12	**0.028**	0.016	0.018	0.012	0.011													
AT_SP14	0.000	0.024	0.000	0.009	0.009	0.018												
MB_SP14	0.022	0.010	0.012	0.020	0.026	0.023	0.006											
P_SP14	**0.031**	0.008	0.010	0.029	0.015	**0.025**	0.002	0.017										
T316_SP14	0.007	0.007	-0.009	0.004	**0.034**	0.018	-0.008	0.010	0.016									
N_SP14	0.016	0.017	0.029	0.018	0.009	0.022	-0.004	0.015	0.002	0.017								
SM_SP14	-0.002	**0.017**	0.019	0.019	0.018	0.010	-0.004	0.007	0.012	0.005	0.003							
V_WP14	**0.179**	**0.227**	**0.220**	**0.208**	**0.208**	**0.155**	**0.188**	**0.204**	**0.208**	**0.222**	**0.200**	**0.168**						
B_WP14	**0.216**	**0.266**	**0.259**	**0.248**	**0.251**	**0.189**	**0.216**	**0.249**	**0.241**	**0.259**	**0.232**	**0.204**	-0.001					
SG_WP14	**0.224**	**0.290**	**0.270**	**0.270**	**0.273**	**0.210**	**0.221**	**0.263**	**0.259**	**0.271**	**0.249**	**0.222**	**0.026**	0.015				
G_WP14	**0.204**	**0.254**	**0.243**	**0.240**	**0.239**	**0.176**	**0.203**	**0.234**	**0.254**	**0.241**	**0.240**	**0.206**	**0.027**	0.032	0.040			
V_WP14	**0.212**	**0.265**	**0.254**	**0.250**	**0.252**	**0.198**	**0.212**	**0.252**	**0.242**	**0.256**	**0.237**	**0.211**	0.004	-0.005	-0.003	0.030		
SG_WP14	**0.209**	**0.255**	**0.242**	**0.236**	**0.239**	**0.176**	**0.203**	**0.236**	**0.241**	**0.240**	**0.232**	**0.201**	**0.022**	0.024	0.007	0.010	0.029	
CG_WP14	**0.190**	**0.227**	**0.223**	**0.211**	**0.213**	**0.149**	**0.196**	**0.217**	**0.218**	**0.219**	**0.214**	**0.183**	**0.014**	0.020	**0.035**	**0.024**	0.020	**0.027**

Chapter 3

Effect of heat waves on embryo mortality in the pine processionary moth

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Effect of heat waves on embryo mortality in the pine processionary moth

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Abstract

Extreme climate events such as heat waves are predicted to become more frequent with climate change, representing a challenge for many organisms. The pine processionary moth *Thaumetopoea pityocampa* is a Mediterranean pine defoliator, which typically lays eggs during the summer. We evaluated the effects of heat waves on egg mortality of 3 populations with different phenologies: a Portuguese population with a classical life cycle (eggs laid in summer), an allochronic Portuguese population reproducing in spring, and a Tunisian population from the extreme southern limit of *T. pityocampa* distribution range, in which eggs are laid in fall. We tested the influence of three consecutive hot days on egg survival and development time, using either constant (CT) or daily cycling temperatures (DT) with equivalent mean temperatures. Maximum temperatures (T_{max}) used in the experiment ranged from 36 to 48ºC for DT and from 30 to 42ºC for CT. Heat waves had a severe negative effect on egg survival when T_{max} reached 42ºC for all populations. No embryo survived above this threshold. At high mean temperatures (40°C), significant differences were observed between populations and between DT and CT regimes. Heat waves further increased embryo development time. The knowledge we gained about the upper lethal temperature to embryos of this species will permit better prediction of the potential expansion of this insect under different climate warming scenarios.

Keywords: climate change, egg tolerance, heat wave, temperature, *Thaumetopoea pityocampa*

Introduction

During the decade of 2002-2011, the global mean surface temperature was 0.77ºC to 0.80ºC warmer than the pre-industrial average; in Europe, the increase reached 1.3°C in terrestrial areas (EEA, 2012). Climatic models predict that global warming will continue in the coming decades. The mean global temperature is projected to increase by 1.1–6.4°C by 2100, while mean temperatures in Europe are expected to rise by 2.5-4.0ºC for the same period (EEA, 2012). Models also predict increased weather variability and increased severity and frequency of extreme weather events such as heat waves, droughts and extreme precipitation (EEA, 2012; IPCC, 2014). These climatic events can have major effects on many organisms. In particular, increasing temperatures are likely to affect the behaviour, development, reproduction, survival and geographical distribution of ectothermic organisms, as their physiological processes are highly dependent on ambient temperatures (Bale et al., 2002; Menéndez, 2007). While an increase in temperature within a favourable range will tend to speed up their metabolism and development, an increase above the optimal threshold will reduce survival or fitness, due to the disruption of metabolic functions (Bale et al., 2002; Chiu et al., 2015). Further, the response of insects to climate depends on the developmental stage and phenology (i.e. the timing of life cycle events) (Bale et al., 2002; Walther et al., 2002; Deutsch et al., 2008). For example, the larval growth can be accelerated by higher temperature whereas the duration of diapause may be extended (Battisti & Jactel, 2010). Most studies on the effects of climate change on species and populations, using e.g. species distribution modeling (SDM) approaches, usually consider mean monthly, seasonal or annual conditions. Similarly, most experimental studies test the consequences of constant temperatures under controlled laboratory conditions. However organisms do not simply experience mean conditions but are exposed to daily fluctuations in temperatures, and a few hours above a lethal maximum can impede species survival or expansion. These daily dynamics have been generally ignored in the climate change literature and could have important effects on the fitness of organisms (Easterling et al., 2000; Walther et al., 2002; Paaijmans et al., 2013).

A heat wave is defined by the World Meteorological Organization and according to the fourth and fifth IPCC assessment reports as a temperature regime in which the daily highest temperatures are 5ºC above the mean maximum temperature for a few consecutive days. Such short-term temperature dynamics can affect life-history traits and fitness beyond the effects of mean temperatures alone (Martin & Huey, 2008;

Bozinovic et al., 2011; Clusella-Trullas et al., 2011; Folguera et al., 2011). The effects of heat waves are particularly severe once they exceed the thermal optima for development, fecundity and/or fitness (Chiu et al., 2015). The negative effects depend on the accumulated damages resulting from exposure to a given temperature. If temperatures are high relative to optimal conditions, even a short exposure can result in substantial mortality. Likewise, longer exposure to high, but not lethal, temperatures may not result in high mortality if individuals take advantage of the lower night temperatures to recover (Mironidis & Savopoulou-Soultani, 2010).

The pine processionary moth *Thaumetopoea pityocampa* (Denis & Schiffermüller 1775) (Lepidoptera: Notodontidae) is the most damaging and widespread defoliator of conifers in the Mediterranean region. Adult emergence, immediately followed by mating and egg laying, occurs in the summer. Eggs take about one month to hatch. The larvae feed on pine needles throughout the fall and winter. In spring, they burrow in the soil to pupate and undergo pupal diapause until the following summer (Démolin, 1969). This phenology, characterized by the development of larvae in winter, is observed in most *T. pityocampa* populations, hereafter called winter populations (WP). An anomalous population displaying a shifted life cycle was discovered in Leiria (Portugal) about 20 years ago. In this spatially localized population, hereafter called SP (summer population), the larvae develop in the summer, pupate in September and the adults reproduce April–May. SP is genetically differentiated from the local sympatric winter population from which it is hypothesized to originate (Santos et al., 2007; Santos et al., 2011a; Burban et al., 2016). Experimental studies showed that this phenology is heritable and possibly originated from a mutation in one or more genes (Branco et al., 2017). As a consequence of this shift in phenology, the different stages of the SP are facing different climatic environments as compared to the WP, with possible implications on climate adaptations as observed on the larval stage (Santos et al., 2011b). We hypothesized that differences in temperature tolerance may also occur in the egg stage.

The relationship between *T. pityocampa* and climate is important as warmer temperatures have led to recent range expansions towards higher latitudes and altitudes in some regions (Hódar & Zamora, 2004; Battisti et al., 2005). This expansion is clearly associated with warming that may improve larval performance and winter survival at the leading, cold edge of the distribution (Hódar et al., 2003; Battisti et al., 2005; Robinet et al., 2007; Hoch et al., 2009). However, increased temperatures can also

produce negative feedbacks, as the heat waves that are predicted to become more frequent may affect eggs and early larval stages during summer and autumn (Robinet et al., 2007). This is particularly relevant in the southern parts of the distribution range, where temperatures are high and might possibly reach lethal limits. *T. pityocampa* eggs and young larvae are sensitive to high temperatures and an excess of heat or exposure to intense solar radiation may induce high mortality (Démolin, 1969). Indeed, a threshold of 32ºC was proposed as a lethal temperature limit for eggs and young larvae by Huchon & Démolin (1970). Consistent with this, the exceptional heat that occurred in Europe during the summer of 2003, with maximum temperatures exceeding 40°C, caused a huge decrease in *T. pityocampa* populations in northern France (Robinet et al., 2013).

Regarding the tolerance of insects to extreme temperatures, few studies investigate the egg stage, most of them focus on the larval stage. Still, while larvae are able to thermoregulate in nature (Kührt et al., 2006) or seek for refugia such as suitable micro-habitats, eggs cannot move or escape, and thus are a highly sensitive stage (Potter et al., 2011). Therefore, gaining knowledge about thermal tolerance of eggs is most important to understand responses to the ongoing climate warming. Robinet et al. (2013) simulated the potential effects of the heat wave of 2003 on *T. pityocampa* egg masses and concluded that a maximum temperature of 40ºC for 5h during 1, 3, 5 and 12 consecutive days had no effects on egg survival. The authors assumed that the possible effect of the 2003 heat wave on *T. pityocampa* populations was due to higher mortality in the young larval stages, which are sensitive to temperatures above 36ºC as demonstrated by Santos et al. (2011b). However, no studies to date have analysed the effects of temperatures above 40ºC on the *T. pityocampa* embryo stage, so the upper temperature threshold for egg survival is still unknown in this species.

Our main goal was here to evaluate the effects of heat wave episodes on egg survival in *T. pityocampa* by testing a large range of maximum temperatures from 36 to 48ºC, thus encompassing the potential range of extreme temperatures that egg masses may experience under current and future climate warming scenarios. We also aimed to test for differences in tolerance of eggs to high temperatures between the unique SP and 2 WP populations from Portugal and Tunisia. The Tunisian population was sampled from a region where extreme summer maximum temperatures frequently exceed 40ºC while the *T. pityocampa* eggs are developing; we therefore expected that the corresponding embryos would be more tolerant to high temperatures. Additionally, considering that

variation in temperature can influence insect survival (Paaijmans et al., 2013), we tested whether egg survival differed between constant temperature regimes (CT) and daily temperature regimes (DT) with equivalent mean temperature. Finally, we wanted to analyse the effect of a pulse of high temperatures on the embryo developmental time.

Material and methods

Sampling of egg masses

In the field, egg laying occurs from the end of April to mid-June for SP, from the beginning of August to September for the Portuguese WP (hereafter PWP) and from mid-September to October for the Tunisian population (hereafter TWP). SP egg masses were thus collected in Nazaré, Leiria (N39º36'50.70"; W9º04'25.80") between the end of May and middle of June; for PWP, egg masses were collected in Pinhal Freiras, Setúbal (N38º34'42"; W9º07'35") in the beginning of September; Tunisian egg masses were collected in mid-September in Djebel Mansour (N36º16'0.0", W9º42'0.0"). Portuguese populations were collected in maritime pine, *Pinus pinaster* Aiton stands whereas Tunisian egg masses were collected in Aleppo pine, *Pinus halepensis* Miller and subsequently transported by plane to Portugal. Egg masses were individually placed in glass test tubes and kept at room temperature (22-28ºC) until trials. Experiments were conducted from 2009 to 2014 for the Portuguese populations and in 2012 and 2013 for the Tunisian population.

The average maximum temperatures for the three sites and periods indicated above are respectively 21.6ºC for Leiria (period May-June), 27.9ºC for Setúbal (period August-September) and 28.5ºC for Djebel Mansour (period September-October), as estimated from weather stations data from the period 1982 - 2012 (WorldClim by Hijmans et al., 2005).

Heat treatments

The tolerance of embryos to high temperatures was tested by exposing egg masses to 3-days of hot temperatures simulated in laboratory-controlled conditions, mimicking heat waves. According to the definition of the IPCC (2014), a heat wave occurs when temperatures exceed the mean maximum temperature by 5ºC during a few consecutive days. A 3-days period was chosen for comparison with previous results on the larval

stage for the same species (Santos et al., 2011b). Two temperature regimes were developed, namely i) daily temperature cycles (DT) and ii) constant heat treatments (CT), using a range of daily average temperatures from 30 to 42°C in both cases. Both CT and DT regimes were implemented in climate chambers Fitoclima S600PL (ARALAB, Portugal), with relative humidity fixed at 60% and photoperiod of 14:10h (L:D). Data loggers OPUS10 were placed inside the chambers to confirm temperature and relative humidity values.

For daily temperature regime (DT), night temperature was fixed 10°C below the maximum temperature (T_{max}) reached during the day. In the morning, temperature gradually increased from the night temperature to T_{max} in 6h, and was then kept constant for the following 4h, corresponding to the expected length of the warmest daily period in the field. Finally, it progressively decreased during the remaining 4h until reaching the night temperature (T_{max} -10°C) (Fig. 1). For clarity, we will hereafter refer to each DT treatment by its daily average temperature, using the codes DT30 (T_{max}=36°C), DT32 (T_{max}=38°C), DT34 (T_{max}=40°C), DT36 (T_{max}=42°C), DT38 (T_{max}=44°C), DT40 (T_{max}=46°C) and DT42 (T_{max}=48°C). Due to small sampling sizes, Tunisian populations were tested only for DT32, DT34, DT36 and DT38.

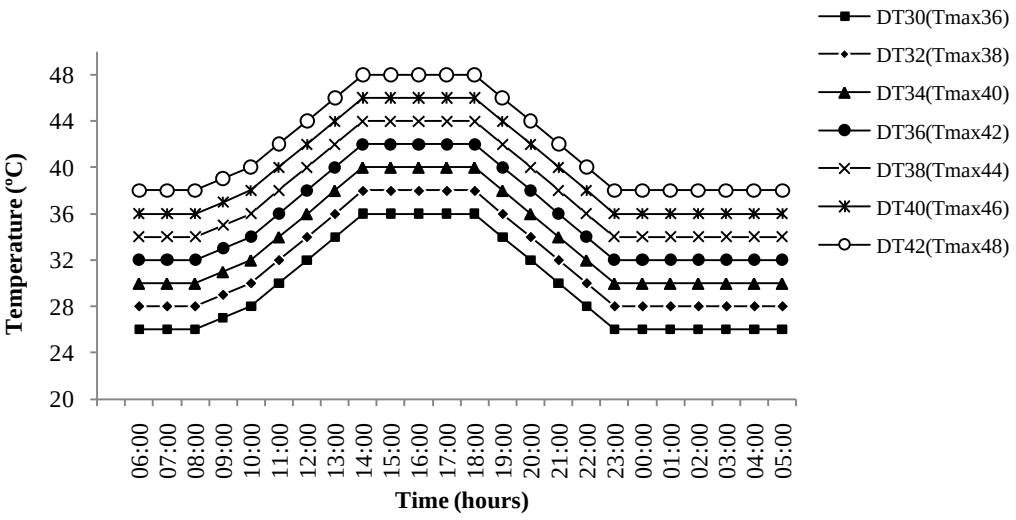

Figure 1 - Temperature daily cycle regimes (DT) simulating heat waves. Each line represents one treatment of fluctuating temperatures with a daily heat peak at maximum temperature T_{max}.. Each cycle was repeated for 3 consecutive days.

Egg masses from SP and PWP were also exposed to constant temperature regimes (CT), which simply consisted of 3 consecutive days at a given temperature, using the same

photoperiod and relative humidity as above. The temperatures selected corresponded to the average daily temperatures used in DT experiments, and were noted CT30, CT32, CT34, CT36, CT38, CT40 and CT42. Number of replicates for each regime and condition are shown in Table 1.

Table 1 - Number of egg masses and average number of eggs per egg mass ± SE used in the temperature experiments for each tested population: Portuguese SP, Portuguese PWP and Tunisian TWP.

	N egg masses			Average N_{egg} ±SE		
Treatment	**SP**	**PWP**	**TWP**	**SP**	**PWP**	**TWP**
25ºC (control)	43	44	28	96 ± 7.6	128 ± 6.5	169 ± 5.5
DT30	16	15	-	116 ± 11.6	139 ± 8.5	-
DT32	20	30	7	113 ± 11.5	158 ± 8.7	160 ± 13.7
DT34	15	25	20	120 ± 12.5	141 ± 9.9	165 ± 9.4
DT36	35	18	18	122 ± 7.1	173 ± 20.9	152 ± 6.5
DT38	15	5	5	120 ± 10.6	108 ± 28.8	168 ± 10.7
DT40	15	6	-	137 ± 6.9	154 ± 15.8	-
DT42	15	7	-	130 ± 7.4	146 ± 13.9	-
25ºC (control)	72	43	-	93 ± 5.4	132 ± 6.2	-
CT30	15	10	-	126 ± 8.8	137 ± 17.8	-
CT32	15	8	-	137 ± 9.2	152 ± 16.9	-
CT34	15	6	-	133 ± 8.0	127 ± 24.6	-
CT36	15	10	-	129 ± 14.5	194 ± 18.9	-
CT38	30	17	-	141 ± 9.7	172 ± 10.9	-
CT40	30	15	-	98 ± 9.4	158 ± 12.4	-
CT42	15	15	-	103 ± 13.6	138 ± 12.2	-

We maximized replicates for controls and for the conditions that proved to be close to the mortality thresholds (see Results).

The experiments were all conducted in Portugal, at the University of Lisbon. They took place in spring for SP, in late summer for PWP and in fall for TWP egg masses, i.e. following their respective sampling periods. For both CT and DT regimes, control groups were held at room temperature (25±2ºC) rather than outdoor conditions to ensure that the control conditions were similar between populations in spite of differences in sampling seasons. Note that 25°C falls within the range of optimal temperature for egg development (Démolin, 1969). In each experimental temperature regime and condition, from 5 to 35 egg masses were tested depending on the number of available egg masses; we used a higher number of replicates for the control (25ºC) because we had to perform several repetitions over several years (Table 1). To allow adaptation from room to higher temperatures, the DT cycle regimes always started from the lower level (night temperature), whereas for the constant regimes CT temperatures increased gradually during 1h until reaching the target temperature. In all cases, after the 3-days treatment, egg masses were placed back at room temperature (~25ºC) and 60% relative humidity until hatching. Hatch date was recorded. Newly hatched larvae were counted, carefully removed, and kept alive in Petri dishes with fresh pine needles for 2-3 days to test their ability to survive after heat treatment. At the end of the experiments, and after a period of at least 50 days in case no eggs had hatched, egg masses were inspected under a binocular microscope (Olympus, SZX12). The number of hatched/unhatched eggs and of parasitized eggs (eggs with a parasitoid exit hole or still containing a parasitoid) were counted. Parasitized eggs were then discounted from the total number of eggs used in any given experiment.

Effect of temperature on the embryo development time

We used egg masses obtained in laboratory conditions, for which we had the exact date of egg laying, to test the effect of different temperature treatments on duration of embryonic development. These eggs were obtained from females resulting from PWP pupae collected in the field that emerged and mated in the laboratory (rearing protocols as described in Branco et al., 2017). Egg masses are difficult to obtain in laboratory conditions, because of a set of constraints such as an obligate univoltine life cycle, a high natural mortality during the larval and pupal stages, the lack of artificial diet, the highly urticating hairs of the larvae and the short life span of the adults and the consequent difficulty of synchronizing emergence to obtain successful mating. Therefore, the numbers of replicates for this experiment were small. The following

treatments were tested: CT36, CT38, CT40 as well DT30, DT32, DT34 and DT36 (N=5 or 4). Control groups were placed at 25ºC (N=9 and N=13, respectively). Age of egg masses varied between 3 and 12 days for this experiment, i.e. they were at 10 to 40% of their embryonic development when tested.

Data analysis

To compare egg survival among the 3 populations (SP, PWP and TWP) and among temperatures for the DT30, DT32, DT34, DT36 treatments, the total numbers of non-parasitized eggs exposed to heat treatment was analysed using a binomial distribution with a logit link function through Generalized Linear Models (GLM). Pairwise comparisons of estimated marginal means based on the events/trials proportion were obtained using least significant differences (α=0.05).

The same procedure was used to compare egg survival between the DT and CT regimes for each population and each tested daily average temperature. First we considered a model with two factors, temperature and regime, but since interaction term was significant, we then used separate models for each temperature. Development time was tested by one way ANOVA after the verification of Levene's test for homogeneity of variances considering the factor temperature. Pairwise comparisons were obtained using least significant differences. All data were analysed with the software program SPSS 22 (SPSS Inc) and using overlap of 95% confidence intervals.

Results

Since egg masses were collected in the field, parasitoids were also recovered. PWP egg masses were the most parasitized mainly by *Baryscapus* sp., with 21±2% of parasitism, followed by TWP with 8±1.1% of parasitized eggs (mean ± S.E.). SP egg masses were practically free of parasitoids (1.8±0.4%) as observed in previous studies (Santos et al., 2013).

Differences between *T. pityocampa* populations in DT

The 3 populations overall differed in their fecundity. Mean number (± SE) of eggs per egg mass was higher for PWP and TWP (147±14.6 and 163±9.2, respectively) than for SP (120±9.6) (Table 1). There were overall significant differences in egg survival between populations (W=286.9, df=2, P<0.001) and among temperatures (W=1165.3,

df= 4, P<0.001). Yet, the interaction term was also significant (W=16.3, df=7, P=0.022). When comparing populations for each tested temperature, TWP had lower survival than both PWP and SP for all temperatures including the control (Table 2). For each population there was a significant effect of temperature (W=375.4, W=389.4 and W=431.2 for SP, PWP and TWP, respectively, P<0.001 in all cases). Still, in all populations, egg survival did not differed significantly from the control (25ºC) for all temperatures up to DT34 (Table 2). A negative effect of temperature was observed at DT36 for all populations, with a higher decrease in survival when compared to control for TWP (about 21%) than for the two Portuguese populations (13% and 9% for SP and PWP, respectively). At DT38, survival was 0% for all populations. For all treatments and populations, the survival of the neonate larvae was 100% during the first 2-3 days, when larvae were fed and observed.

Table 2 – Embryo survival (average ± SE) of Portuguese SP, Portuguese PWP and Tunisian TWP populations exposed to the daily cycle temperature (DT) regimes with maximum temperatures (T_{max}) of 36, 38, 40, 42 and 44ºC as well as the control at 25ºC constant temperature.

	SP	PWP	TWP	*P-value*
25ºC (control)	$96\pm0.3^{a, A*}$	$95\pm0.3^{b, A}$	$91\pm0.4^{c, A}$	<0.001
DT30 (T_{max}=36)	$97\pm0.4^{a, A}$	$94\pm0.5^{b, A}$	-	<0.001
DT32 (T_{max}=38)	$97\pm0.4^{a, A}$	$95\pm0.3^{b, A}$	$92\pm0.8^{c, A}$	<0.001
DT34 (T_{max}=40)	$96\pm0.5^{a, A}$	$96\pm0.3^{a, A}$	$91\pm0.5^{b, A}$	<0.001
DT36 (T_{max}=42)	$87\pm0.5^{a, B}$	$83\pm0.8^{b, B}$	$72\pm1.2^{c, B}$	<0.001
DT38 (T_{max}=44)	0	0	0	

*Different lowercase letters indicate significant differences between populations
Different uppercase letters indicate significant differences between DT regimes

Cycle versus constant temperature effect

Differences between CT and DT conditions corresponding to the same daily average temperatures were tested in the two Portuguese populations. Up to an average temperature of 34°C (CT34 and DT34), survival was very high (>90%) and was not significantly different between the two regimes (Fig. 2, Table 3), nor different from the control. A negative effect of heat treatment started in the pair CT36/DT36 with a similar decrease in survival for both DT and CT regimes for each population (Fig. 2). Although

egg survival decreased for both populations in these trials, the mortality was higher for PWP (Fig. 2). Still, for the pairs CT38/DT38 and CT40/DT40 significant differences were found between the two regimes (Table 3). For both DT38 and DT40 survival was 0% for both populations, while for the equivalent CT38 and CT40 egg survival was observed to be 70±0.9% and 38±1.0% in the case of PWP, and 70±0.7% and 1±0.1% in the case of SP, respectively (Fig. 2). For DT42 and CT42, mortality was 100% for both populations.

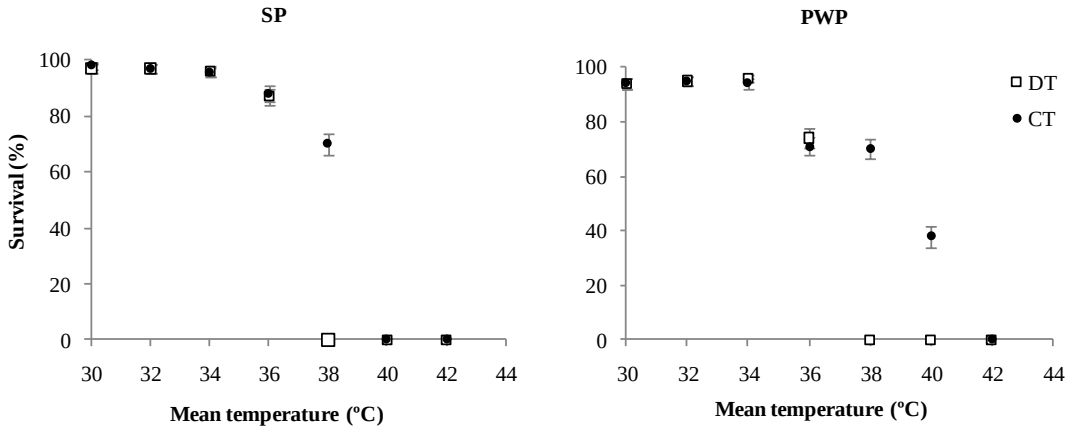

Figure 2 - Embryo survival (average ± 2SE) in two experimental regimes: daily cycles (DT) vs constant temperatures (CT) for two Portuguese populations, SP and PWP. Horizontal axis, mean temperature corresponding to maximum temperatures (T_{max}) for daily cycles as follow: DT30 (T_{max}=36), DT32 (T_{max}=38), DT34 (T_{max}=40), DT36 (T_{max}=42), DT38 (T_{max}=44), DT40 (T_{max}=46), DT42 (T_{max}=48).

Table 3 - Comparison of embryo survival when egg masses were exposed to constant temperature (CT) and daily cycles (DT) regimes, for Portuguese SP and Portuguese PWP populations.

Cycle (Constant)	SP	PWP
DT30 (CT30)	W=2.448; P=0.118	W=0.120; P=0.729
DT32 (CT32)	W=0.034; P=0.854	W=0.000; P=0.984
DT34 (CT34)	W=0.200; P=0.655	W=1.917; P=0.166
DT36 (CT36)	W=0.476; P=0.490	W=3.000; P=0.083
DT38 (CT38)	W=9714.8; P<0.001	W=6810.3; P<0.001
DT40 (CT40)	W=16.10; P=0.001	W=1434.4; P<0.001
DT42 (CT42)	*	*

* No embryo survived at this temperature regime.

While living adult parasitoids were found in all treatments, the egg masses subjected to the highest temperature also hosted dead adult parasitoids. The maximum temperature treatments for which parasitoids emerged were CT38 (9 *Ooencyrtus* sp. and 26 *Baryscapus* sp.) and DT36 (1 *Ooencyrtus* sp. and 14 *Baryscapus* sp.). Since we could not control the number of parasitoids in each regime, it was not possible to statistically test the effects of temperature on the survival of the egg parasitoids.

Effect of temperature on the embryo development time

In the constant regime, the CT36, CT38 and CT40 conditions all had a significant effect on the egg developmental time causing an increased delay in hatching with increased heat treatment in comparison with the control (Table 4). A similar increase in development time was observed when eggs were submitted to DT32, DT34 and DT36 (Table 4). The longest embryo development time was observed for DT36 with a mean of 35 days (Table 4), i.e. 5 days longer than the mean development time at 25ºC.

Table 4 – Embryo development time (average ± SE) (days) of Portuguese PWP egg masses exposed to constant (CT) and daily cycle (DT) temperature regimes, at different maximum temperatures (T_{max}).

CT regime (T_{max})	Development time (days)	N
25ºC (control)	30.1±0.2^{a}	9
CT36	32.0±0.3^{b}	4
CT38	32.6±0.3^{bc}	5
CT40	33.8±0.3^{c}	4
CT42	*	
$F_{3,18}$=29.82; *P<0.001*		
DT regime (T_{max})	Development time (days)	N
25ºC (control)	29.4±0.2^{a}	13
DT30 (T_{max}=36)	30.0±0.3^{a}	5
DT32 (T_{max}=38)	31.0±0.3^{b}	5
DT34 (T_{max}=40)	31.6±0.3^{b}	5
DT36 (T_{max}=42)	35.0±0.4^{c}	4
$F_{4,27}$=48.79; *P<0.001*		

Discussion

Identification of upper lethal temperatures for *T. pityocampa* embryos

Many studies have demonstrated the detrimental effects of high temperatures on insect survival. Yet, the threshold limits are species and stage specific (Chiu et al., 2015), and can only be identified through stage-specific experimental studies for each species. Most of the studies conducted thus far concern the larval, nymphal or adult stages and only a few of them have explored the egg stage. These studies demonstrate large differences among species in their sensitivity to temperature. For example, in a study of the effect of high temperatures on mortality of different life stages of the almond moth *Ephestia cautella* (Lepidoptera), Darwish et al. (2015) showed that the egg stage was the most sensitive to high temperatures and that an exposure to 45ºC for 59 min or more caused 95% egg mortality. In a study conducted with *Parahypopta caestrum* (Lepidoptera), Salpiggidis et al. (2004) found that eggs were already negatively affected at 35ºC.

For *T. pityocampa*, we observed a severe decline of embryo survival when T_{max} reached 42ºC, and 100% mortality above this threshold. On the other hand, up to T_{max} = 40ºC, the survival of the heat-treated groups did not differ from the controls kept at 25ºC. In the study conducted by Robinet et al. (2013), the authors concluded that a heat wave corresponding to a maximum of 40ºC (T_{max}) for 5 hours a day over 12 days had no effect on *T. pityocampa* egg survival, which is consistent with our findings. Using higher temperatures, we could identify the upper lethal temperature for *T. pityocampa* egg stage. This limit is situated near 42ºC, and affects egg survival after only 3 days. Interestingly, this temperature is well above of the 32°C that Huchon & Démolin (1970) reported as the lethal temperature of eggs and young larvae. Differences between this past study, in which no clear protocol was described, and recent ones could be attributed to different methodologies, to differences among populations or possibly to adaptive evolution that could have occurred in *T. pityocampa* populations during the past 40 years as a consequence of climate warming, as already suggested in Santos et al. (2011b).

We showed that the lethal temperature threshold for *T. pityocampa* embryos is relatively high, which is consistent with the Mediterranean origin of the species (Simonato et al., 2013). Temperatures above 42ºC are not frequent but can be sometimes observed in the studied area in very hot summer years (Santos et al. 2011b). Further, *T. pityocampa* females tend to lay their eggs exposed to the sun, and since temperature below the

scales of the egg masses may be higher than the air temperature (Robinet et al., 2013), an exposure to temperatures above 42ºC can occur even when air temperatures under shelter remain below that threshold. Lastly, as we could not precisely control the age of the egg masses sampled in the field, we cannot rule out the hypothesis that embryos at the very beginning or end of development would be sensitive to lower temperatures than the threshold obtained from our results, and that we tend to underestimate the negative effect of heat waves. Thus, egg mortality due to heat waves may occur in the studied region and become even more frequent in the future according to climate change scenarios (EEA, 2012).

Daily cycling vs. constant temperature regimes

Most studies on the effects of climate change consider mean temperatures or mean maximum values, and do not consider that a short exposure (few days or few hours) above a certain temperature can cause high mortality. Experimental protocols using constant temperatures are often chosen because they are easy to handle in the laboratory. Yet, they are likely to result in a number of biases (Robinet & Roques, 2010). Moreover, changes in mean temperatures do not reflect those occurring in maximum and minimum temperatures, which can affect survival and development and could hinder any advantages offered by a warmer mean temperature in temperate environments (Vasseur et al., 2014). Our study showed that both CT and DT regimes induce low mortality until a certain threshold is reached. On the other hand, the two regimes differ near the upper limit. For example, for a similar daily mean temperature of 38°C, no embryo survived at DT38 for both populations while survival was still high (70%) for both populations in the constant CT38 experimental condition. Thus extreme values of both mean and maximum daily temperatures and their probability of occurrence in a given environment should be taken into account in probabilistic models of species distribution, as both can affect the probability of local extinction.

Temperature thresholds differ among populations

The 3 populations differed in egg survival. For all temperatures tested, including the control, embryo survival was highest in SP and lowest in TWP, while differences between SP and PWP were minor. Since the differences among populations were also significant for the control (25°C), they are possibly explained by factors besides the

experimental treatment. The larger egg size of SP, and thus the greater amount of available resources (Santos et al., 2013), could explain why embryo survival was highest for this population. In the case of the Tunisian population we assume that their transport conditions, including airplane travel, might have partially affected egg viability. Embryo tolerance to temperature could also be affected by egg age, which might have influenced the results but cannot explain differences observed for the control group. On the other hand, host trees differed between Tunisia and Portugal; we may assume that host food quality of the mother female affect embryo survival. Studies using similar host tree species for all populations would be needed to test this hypothesis. However, other factors than pine species could play a role to possibly change egg mass characteristics in the two populations.

In the CT experiments, we found that PWP embryos were more tolerant to high temperatures than those of SP, as 38% of PWP eggs could survive to a constant 3-day temperature of 40ºC whereas all SP embryos died under these same conditions (Fig. 2). This may be explained by the fact that PWP eggs are laid during summer and exposed to daily maximum temperatures that may exceed 40ºC. On the contrary, SP eggs are laid during the spring and are typically not exposed to such high temperatures. On the other hand, results were similar for both populations under DT regimes, suggesting that both populations can tolerate similar maximum temperatures as long as those occur only few hours a day.

T. pityocampa exhibits variable phenology across its distribution range with moths emerging later (after the warmest temperatures) in the southern and lower-altitude areas, and earlier (before the warmest temperatures) in the northern and high-altitude regions (Démolin, 1969; Huchon & Démolin, 1970). Our results suggest that high summer temperatures can affect embryo survival, and probably impose strong natural selection. High mortality due to hot summer temperatures of both eggs (this study) and neonate larvae (Santos et al., 2011b) would then favour the natural selection of late-emerging individuals in regions where summer temperatures are high, possibly through the evolution of longer pupal diapause. Such selection pressure could be especially strong because each individual only lives for 1-3 days and thus reproduces immediately after adult emergence (Branco et al., 2017). Plastic or adaptive changes in phenology could thus be a way to overcome seasonal unfavourable conditions, and could be an efficient response to climate change for many organisms (Bale et al., 2002; Root et al., 2003; Musolin et al., 2010; Clusella-Trullas et al., 2014; Robinet et al., 2015).

Other mechanisms, such as physiological and biochemical adaptations, can allow organisms to overcome unfavourable temperatures (Denlinger & Yocum, 1998; Angilletta et al., 2002). Studies looking for differential expression of target genes in eggs and larvae exposed to high temperatures might increase understanding of the underlying metabolic mechanisms. Such a perspective is now feasible thanks to the recent development of transcriptomic and genomic resources (Gschloessl et al., 2014).

Development time

Although higher temperatures usually speed up metabolism and development in poikilothermic organisms, short exposure to extreme high temperature might have an opposite outcome. Indeed, we observed that egg development time increased with heat treatment, independently of the temperature regime applied (daily cycle or constant), at least when using between 3 and 12-days old embryos. For DT36, the time of egg development was almost 5 days longer than the control (25ºC), and for CT40 eggs took almost 4 more days to hatch than the control. Similarly, longer egg development times with increasing temperatures and exposure time were observed in other Lepidoptera, namely *Helicoverpa armigera*, *H. punctigera* (Qayyum & Zalucki, 1987) and *Manduca sexta* (Potter el al., 2011). Such results suggest a further negative effect of high temperatures by indirectly increasing exposure to natural enemies during the egg stage. However, it might also be adaptive, as such a delay allows the young larvae to hatch further in time from the heat wave, which would increase the survival of this highly sensitive stage. In fact in a previous study it was found that significant mortality of PWP young larvae occurs at T_{max}=38°C (Santos et al., 2011b), whereas the threshold found here for the embryo was T_{max}=42°C.

Parasitoids

According to Hance et al. (2007), both hosts and parasitoids are affected by extreme temperatures, and any effect on the host has consequences for the parasitoids within. Still, it is possible that parasitoids respond differently to extreme conditions and could have different temperature threshold for survival. This is a relevant issue since different effects of climate changes on the phenology or survival of the two trophic levels might have consequences for their population dynamics by disrupting the biotic interactions. In our study it was not possible to follow the effects of high temperatures on egg

parasitoids despite their ecological interest, because parasitoid survival rates could not be estimated and the parasitoid stage that experienced the heat conditions could not be controlled. However, we did observe that a few parasitoids survived in the CT38 and DT36 conditions. Although these results are only preliminary, it seems that parasitoids may cope, at least partially, with these high temperatures.

Conclusions

Our study tested the effect of a wide range of high temperatures and allowed setting the lethal egg temperature for *T. pityocampa* in the case of short-term exposures. Further, differences were registered between a population with spring egg phenology (SP) and the typical PWP population with summer egg laying. A significant effect of simulated heat waves on egg developmental time was also observed. Considering potential effects of future climate warming scenarios, present findings suggest that environmental conditions in the southern regions may become less favourable to *T. pityocampa* populations that have the typical winter phenology due to the expected increase of summer temperatures. Shifts in phenology towards even later emergence may be further expected. Concerning the evolution of the SP, even though its embryo survival is not expected to be affected by spring temperatures, as they are likely to stay below the detrimental threshold, its fate under climate change may be threatened due to increased mortality of neonate larvae in summer (Godefroid et al., 2016). It is similarly possible that the current expansion of *T. pityocampa* observed at the northern edge of its distribution could be impeded if the frequency of summer heat waves increases as expected. Taking into account the intensity and the probability of occurrence of heat waves will be necessary in the future to forecast the evolution of populations under climate change.

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CHAPTER 4

Shifted phenology in the pine processionary moth affects the outcome of tree-insect interaction

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Shifted phenology in the pine processionary moth affects the outcome of tree-insect interaction

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Abstract

In Mediterranean and temperate regions, an increase in the frequency and intensity of drought events has been recorded, probably due to climate change. In consequence, trees will more frequently experience hydric stress, a condition that can be expected to affect insect-tree interactions, while adaptation mechanisms may be further in course. The effect of tree water stress on the performance of two allochronic populations of *Thaumetopoea pityocampa* was here studied. Namely, we compared a unique population of this insect, in which the larvae develop in the summer (SP), with the typical population having winter larval development (WP), to test the adaptation hypothesis to host plant status. Larvae of each population were fed on needles of young potted *Pinus pinaster* plants under two water supply regimes: (i) well-watered (control) and (ii) subjected to 3 months of drought stress. Compared to control, stressed plants had higher amounts of soluble sugars, phenols and higher C/N ratio, whereas water content and chlorophylls concentrations were lower. In general, *T. pityocampa* larvae had lower performances on water stressed plants, as shown by lower survival rates, lower needle consumption and longer development times. Yet, the detrimental effects of tree stress were only significant for the WP larvae, while SP larvae were able to overcome such conditions. Results demonstrate that tree water stress can negatively affect *T. pityocampa* populations. Furthermore, evidence is also provided that responses to the physiological condition of the host trees may occur at population level, as a result of adaptation mechanisms driven by climate change.

Keywords: climate change, drought stress, intra-specific variation, pine processionary moth, plant-insect interaction

Introduction

Global climate change, characterized by increased temperatures and temporal variability of precipitation (IPCC, 2013), is resulting in a higher frequency and intensity of drought events (Hoerling et al., 2012). In particular, prolonged summer/autumn droughts are predicted to occur more often in Mediterranean regions (IPCC, 2007). Warming and drought decrease soil water availability and increase atmospheric evaporative demand, leading to growth and physiological changes in water stressed trees (McDowell et al., 2008). Effectively, in many regions, increased tree mortality is often associated with drought, and sometimes jointly with insect outbreaks (Anderegg et al., 2015). However, although demographic increase of insect populations can result directly from positive effects of temperatures on development rates or survival (Ayres & Lombardero, 2000) it is not so straight forward to predict how changes in host trees due to drought may affect insect populations (Jactel et al., 2012; Anderegg et al., 2015).

Tree responses to water stress can alter their attractiveness and physical and nutritional value for herbivorous insects (Mattson & Haack, 1987; Jaleel et al., 2009). For example, water stress may reduce tree tissue water content and turgor, decrease transpiration and photosynthesis leading to the accumulation of osmolytes such as amino acids and carbohydrates (Rouault et al., 2006; Netherer & Schopf, 2010) that can increase the performances of herbivorous insects (Mattson & Haack, 1987; Caldeira et al., 2002). On the other hand, secondary metabolites such as phenols may increase in water stressed trees, then negatively affecting the performance of herbivorous insects (Koricheva et al., 1998; Huberty & Denno, 2004; Kolb et al., 2016).

Experimental evidence has shown that insect performance may increase (Mattson & Haack, 1987), decrease (Huberty & Denno, 2004; Branco et al., 2010) or remain unaffected (Koricheva et al., 1998; Huberty & Denno, 2004) in response to water stressed host trees. Numerous studies have suggested that different insect feeding guilds could show different responses to drought stress, due to different evolutionary and physiological mechanisms (Koricheva et al., 1998; Inbar et al., 2001; Huberty & Denno, 2004; Rouault et al., 2006). Generally, bark beetles and wood borers benefit from severe drought (Koricheva et al., 1998; Huberty & Denno, 2004), sap suckers (e.g. aphids) are impaired (Huberty & Denno, 2004; Rouault et al., 2006), whereas the effect on leaf miners, leaf chewers and gall makers is more uncertain (Larsson, 1989; Koricheva et al., 1998; Huberty & Denno, 2004). More, intra-specific differentiation and adaptation mechanisms at population level can modify locally the interactions between herbivorous

insects and water stressed trees. Recent works have highlighted adaptation mechanisms at population level, due to selective pressures in regard to environmental changes, concerning, for example, the response to temperature (Santos et al., 2011b). Phenotypic traits associated with plant feeding, such as the size of mandibles and their strength (Zovi et al., 2008), or physiological mechanisms allowing insects to cope with plant chemicals (Després et al., 2007) may be under strong selective pressure in response to changes in host plants. Within the context of climate change, it is critical to understand if and how insect species are able to cope with drought-stressed hosts (Kolb et al., 2016), and if the outcome of such interactions can vary depending on the pest population considered.

Among defoliators, *Thaumetopoea pityocampa* (Denis & Schiffermuller) (Lepidoptera: Notodontidae), the winter pine processionary moth (PPM) is one of the most damaging defoliators of conifers in Europe, and a threat to human health (Battisti et al., 2017). The species has one generation per year. Over its wide distribution range across the Mediterranean Basin, adult emergence, mating and egg laying normally occur in the late summer months. Larvae hatch about one month later and live gregariously for several months. They feed on pine needles, preferentially the mature ones grown during the previous years (Jacquet et al. 2014), throughout the fall and winter. By late winter/early spring, they leave the nest and search for a pupation site in the soil. Adult emergence occurs the following late summer months (Démolin, 1969). This phenology, characterized by the development of larvae in winter, is typical of the PPM throughout its range, and correspond to the so-called "winter populations" (WP). This typical *T. pityocampa* phenology is modulated accordingly to climate along its latitudinal and altitudinal range, still keeping summer oviposition. By contrast, a unique population displaying a shifted life cycle, in which adults emerge in the spring and the larvae develop throughout the summer (Santos et al., 2007) forming the so-called summer population (SP), was discovered in Portugal (in Mata Nacional Leiria - MNL) ca. 20 years ago. This SP population is genetically differentiated from the local sympatric WP from which it is hypothesized to originate (Santos et al., 2007, 2011a; Burban et al., 2016). The two sympatric populations are reproductively isolated by time as adults of the two populations have non-overlapping, heritable periods of activity (Branco et al., 2017). It represents one of the best documented cases of true allochronic speciation (Taylor & Friesen, 2017).

Since it was discovered, the SP has been expanding to the south, north and east of the core MNL area (Godefroid et al., 2016). In this region, SP and WP populations co-occur in the same stands, feeding on the same host plants (*Pinus pinaster* Aiton). In consequence of this shift in the phenology, the different developmental stages of the SP are subjected to different climatic conditions as compared to the WP. In particular, SP larvae develop under high summer temperatures, when trees are generally water stressed, while WP larvae occur in fall and winter, when temperatures are much lower and rainfall is frequent (Fig. 1).

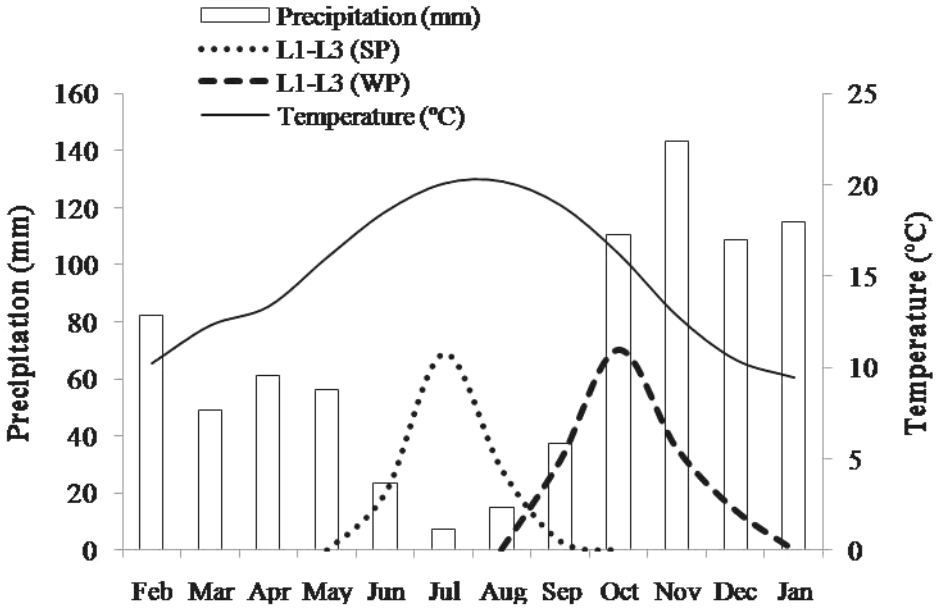

Figure 1 – Distribution of rainfall and mean monthly temperature in the studied area (meteorological station of Monte Real, Leiria, data for the period 1986-2016). Distribution curves of the young larval stages (L1 to L3) for the two populations, SP and WP, based on the respective males flight period (Santos et al., 2011a); the embryonic development (ca. 1 month) and the total period for L1, L2 and L3 development (ca. 2 months).

In this study, we capitalize on the occurrence of the two genetically differentiated populations of PPM, to investigate potential adaptive response to host mediated-water stress. To achieve this, we first analyzed the effect of drought on needle chemical and physical characteristics, and then compared the performance of the two PPM populations on control and water stressed host trees during the first larval stages.

We hypothesize that SP larvae can feed more efficiently on tougher needles, and thus perform better than the WP larvae on water stressed pines. To test our hypothesis we developed an experimental design in order to (i) determine if larval performances were affected by a moderate water stress of the pine host, and (ii) compare the capacity of the two PPM populations to develop on drought stressed trees, which may underlie adaptation mechanisms in the context of climate change scenarios.

Material and methods

Tree material and treatments

Five year-old trees of *P. pinaster*, about 1.8 m high, obtained from a forest nursery (Aliança Florestal, Portugal) were transplanted to pots (volume 25 L, height 35 cm) filled with a substrate of turf, vermiculite 2:1 (v/v) and nutrients (Projar Profissional). During two months trees were kept outside, well-watered and fertilized weekly (NPK 4:3:6 and micronutrients) (Bio-Grow Biobizz) until the experiments started.

Trees were then transferred to a greenhouse and randomly assigned to two different hydric treatments: (i) non-limiting water input (control) and (ii) moderate water stress. Ten replicates per treatment were used. As the larvae of the two *T. pityocampa* populations develop in different seasons, the feeding experiments were performed accordingly in July-August 2015 for the SP trial and in October-November 2015 for the WP trial. For both trials the onset of the tree water stress started 3 months before larval hatching (i.e., in April 2015 for the SP trial and July 2015 for the WP trial). Plants were kept in greenhouse conditions: 14 h/10 h (light/dark), mean air temperature (± s.e.) 26ºC ± 2ºC, relative humidity 50% ± 2%, during the SP trial; and 12 h/12 h (light/dark), 23ºC ± 2ºC and 60% ± 2% during WP trial.

Water stress was imposed through the incomplete replacement of the water transpired from the plant, which was estimated by weighting the tree pots weekly. Replacement of the water lost was achieved by successively reducing this amount by 10% per week, until moderate water stress was attained as defined by the leaf water potential, and then maintained at that level. Water from the pots was only lost through tree transpiration as the top of the pots was closed with a white plastic sheet to avoid direct evaporation from the soil. Well-watered trees were watered to soil field capacity once or twice per week, depending on air temperature. Air temperature and relative humidity (reference sensor)

inside the greenhouse were continuously monitored with an Em50 datalogger (Decagon Devices Inc, Washington, USA).

Plant water status

In both SP and WP trials, tree water condition was monitored by measuring the predawn needle water potential (Ψ_{pd}) in a pair of one-year-old needles (i.e grown the previous year) for all trees, using a Scholander pressure chamber (PMS-1000, PMS Instruments, Corvalis, Oregon, USA). The water potential was evaluated twice for each trial, one month previously to the beginning of the test and at the start of the feeding experiments. Tree water status was also determined by measuring the needles relative water content (RWC), a parameter that reflects the metabolic activity in tissues and is used as an index for dehydration tolerance (Anjum et al., 2011). Ten needles per tree were collected at dawn and immediately weighted to obtain their fresh weight (FW); next they were placed in distilled water for 24 h in the dark, to achieve turgid weight (TW) and finally samples were dried at 80ºC for 48 h to obtain their dry weight (DW). RWC values were calculated as: $RWC = (FW - DW) / (TW - DW) \times 100$.

Foliar analyses

We assessed relevant needle biochemical (chlorophyll, soluble sugars, phenols, carbon and nitrogen) and physical (hardness) characteristics using one-year-old fresh needles from controlled and stressed trees from SP and WP trials. The needles for chemical analysis were collected when feeding trials started, and were stored at -80ºC. For chlorophyll and soluble sugars, three samples from each tree were analyzed from different tree canopy expositions, while for phenols, carbon and nitrogen, one composite sample per tree, comprising needles collected from different tree orientations was used. Still, in both cases, representative samples were obtained for each tree.
Chlorophyll content (*a*, *b* and total) was determined using a modified version of Arnon (1949) method. Extractions were made from 0.1 g of fresh material with ethanol 80% (v/v). After centrifugation at 13000 rpm for 5 min, absorbance was read at 663 and 645 nm for chlorophyll *a* and *b*, respectively. Soluble sugars (glucose, fructose and sucrose) were measured as described by Stitt et al. (1989) based on the enzymatic method of Jones et al. (1977) using 0.1 g of fresh needles. Total phenols content were determined based on the Folin-Ciocalteu method (Folin & Ciocalteu, 1927) using 0.5 g of dried (48

h at 60ºC) fine powered needle. Calibration curves were created using standard gallic acid solutions and the amount of total phenols was expressed in mg of gallic acid equivalents per g of dry weight.

Total needle nitrogen concentration was analyzed by a modified Kjeldahl analysis (Berthelot reaction, modified) (Searle, 1984) using 0.5 g of fine milled needles. Samples were digested with 5 ml sulfuric acid (98 %) and selenium as a catalyst for 3 h at about 350°C. On cooling, it was diluted to 50 ml with distilled water and shaken thoroughly. Quantifications were made by molecular absorption spectrophotometry in the visible region (660 nm). The N-concentration of the digests was determined on a continuous flow analyzer (Skalar SanPlus System, Breda, The Netherlands), while total needle carbon (C) was determined by quantification of CO_2 by infrared after combustion at 1200°C (Analytik Jena EA 4000, Jena, Germany). Results were expressed as percentage of dry matter in both cases, and the C/N ratio was determined.

Needle toughness was measured with a penetrometer that determined the strength necessary to rip the needle epidermis, as an indicator of the strength that larvae would need when feeding on the needles. Ten pairs (1-year old) needles per tree for each plant treatment and trial (SP and WP), were measured for toughness in the middle of the needle top surface. Values were expressed in units of force (Newton).

PPM population feeding trials

Larvae of the two PPM populations were obtained from egg masses collected in the field, from *P. pinaster* trees. For SP populations, egg masses were collected at the end of June, in Nazaré (39º36'50.70"N; 9º04'25.80"W), and for WP, collections were made at the end of September, in Setúbal Península (38º34'42"N; 9º07'35"W). Egg masses were individually placed in glass test tubes and kept at room temperature (22ºC to 28ºC) until larval hatching. For both populations, 30 newly hatched larvae were used in the trials that started at the beginning of July for SP and at the end of September for WP. The feeding experiments were carried out in glass Petri dishes (140 mm in diameter) where larvae were fed, following the feeding protocol described in Hódar et al. (2002). Each Petri dish corresponded to one tree of each treatment. In total, each PPM population feeding trial consisted of 10 replicates per treatment. At each time, 4 pairs of needles (1 year old), inserted into wet floral foam to avoid excessive loss of water, were offered to the larvae. The Petri dishes were placed in rearing chambers with controlled temperature (25 ± 2ºC) and relative humidity (60 ± 2%) within the range of optimal

conditions for larval development (Démolin, 1969), at the entomological laboratory of Forest Research Centre, Lisbon University. Larvae were inspected every 3-4 days for food replacement, cleaning, feces removal and counting of dead larvae. Larval performance was estimated by larval survival, consumption rate, feces production and development time (number of days to reach the 2nd instar) for each treatment. The development time until 3rd instar was not estimated due to the low number of individuals reaching this stage, especially on the WP trial. The consumption rate was determined by measuring the total length (mm) of needles consumed. Feces production was calculated based on the feces dry weight. Both consumption and feces produced were then divided by the number of individuals alive at each sampling date, to obtain average values per individual. The feeding trials were conducted until larvae reached the 3rd instar, after which they become urticating and thus difficult to handle.

Statistical analyses

Independent sample t-tests were performed to compare needle water potential between treatments (stressed and control), for each date of measurement. Levene's test was used to test for equal variances between groups. Whenever equal variances were not assumed, degrees of freedom were adjusted. RWC, chlorophylls, soluble sugars and toughness were analyzed through Generalized Estimating Equations (GEE), with normal distribution and identity link function. Each tree was a replicate and needles from the same tree were repeated measurements accounting for within-subject effect. Consumption and feces production by the larvae were also analyzed through GEE, with normal distribution and log link function. Each rearing Petri dish was a replicate and observations on different dates were considered repeated measurements. Generalized linear models (GLM) with log link function were used to test for differences between treatments of C/N values and larvae development time. A two-way ANOVA was used for analyzing the effects on total phenols. For all models, the factors tree treatment and trial were considered as categorical predictable variables. Linearly independent pairwise comparisons among the estimated marginal means were conducted using Least Significant Difference test. For GLM and GEE models, statistics are presented in the form of Wald Chi^2. Degrees of freedom were 1 in all cases. The mean number of days of larval survival for each trial was estimated by Kaplan Maier method. Individuals alive at the end of the period were used as censored ones. The Log Rank (Mantel-Cox)

test was used to compare mean survival between groups of larvae from different treatments. Statistical analyses were conducted with IBM SPSS statistics 23 software.

Results

Tree parameters

Two months after the stress treatment started, that is one month before the beginning of the feeding trials, the needle predawn water potential (Ψpd) was already significantly lower in stressed than in control trees, for both SP and WP trials (Fig. 2) (Trial SP: T-test = -7.344, df = 10.52, $P < 0.001$; Trial WP: T-test = -5.120, df = 9.98, $P < 0.001$).

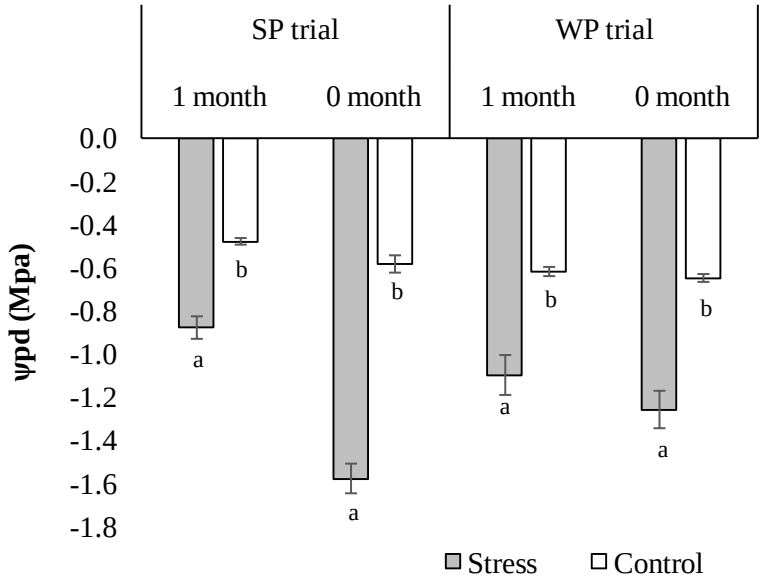

Figure 2 - Predawn needle water potential (Ψpd, MPa), (mean ± SE), on drought stressed and control trees, of trials SP and WP, measured one month and just (0 month) before the larvae feeding experiment started.

Differences increased until the onset of the feeding trial (0 month) (Fig. 2) (Trial SP: T-test = -12.489, df = 18, $P < 0.001$; Trial WP: T-test = -6.873 df = 9.79, $P < 0.001$). Levene's test further evidenced unequal variance with higher variance on the stressed trees, in comparison with control ones for most cases.

Accordingly, needles RWC was higher for control (Trial SP = 94.4 ± 0.25%; Trial WP = 92.7 ± 0.17%) than for stressed trees (Trial SP = 80.9 ± 0.16%; Trial WP = 77.6 ±

0.52%). Differences between tree treatments, considering both trials, were statistically significant (Wald $Chi^2 = 2107.193$, df = 1, $P < 0.001$).

Tree treatment produced significant effects on all biochemical compounds analyzed, as well as on needles toughness (Table 1, Table 2). Consistently, total soluble sugars, phenols and toughness were higher in water stressed trees, in both trials. An opposite trend was found for chlorophylls, which were higher on control trees than on stressed ones (Table 2). The effect of trial (SP or WP) was only significant for the carbon/nitrogen ratio and not for the remaining parameters (Table 1). C/N was higher on stressed trees but differences were only significant for the SP trial. The interaction trial x treatment was never significant (Table 1). Total soluble sugars were represented mainly by sucrose accounting for 67% and 74% in control and stressed trees, respectively. Overall, glucose accounted for only 6% and fructose to 23.5% of total soluble sugars content.

Table 1 - Results of statistical analysis on needles' traits from two treatments (stressed and control trees) and two trials (SP and WP). For C/N, sugars, Chlorophylls and Toughness, statistical test corresponds to Wald Chi^2 (df = 1) obtained with Generalized linear models; for phenols, a two way ANOVA was used ($F_{1,36}$). For all models the factors treatment (Treat.), trial and its interaction term were considered.

Needles Trait		**C/N**	**Sugars**	**Chlorophylls**	**Phenols**	**Toughness**
Treat.	Test	4.772	12.640	9.853	14.532	36.456
	P value	*0.029*	*< 0.001*	*0.002*	*0.001*	*< 0.001*
Trial	Test	12.373	1.125	1.018	0.199	1.108
	P value	*< 0.001*	0.289	0.313	0.658	0.293
Treat.	Test	0.517	0.070	0.277	1.713	1.353
x Trial	*P* value	0.472	0.791	0.598	0.199	0.245

Table 2 - Physical and chemical parameters analyzed on stressed and control trees and on SP and WP trials (mean ± standard error). Different letters mean statistically significant differences between treatments determined by multiple pairwise comparisons through Least Significant Difference (α=0.05).

Trial/ Treatment		C/N	Sugars µmol.g^{-1}	Chlorophylls (mg.g^{-1})	Phenols (mg.g^{-1})	Toughness N (x100)
SP	control	0.94 ± 0.08 ^{a}	15.6 ± 0.5 ^{a}	0.77 ± 0.03 ^{a}	51.6 ± 2.4 ^{a}	3.47 ± 0.15 ^{a}
	stress	1.14 ± 0.07 ^{b}	19.4 ± 1.2 ^{b}	0.66 ± 0.03 ^{b}	61.6 ± 3.9 ^{b}	4.01 ± 0.11 ^{b}
WP	control	1.24 ± 0.09 ^{b}	16.9 ± 0.6 ^{a}	0.72 ± 0.03 ^{a}	48.1 ± 4.6 ^{a}	3.22 ± 0.10 ^{a}
	stress	1.37 ± 0.08 ^{b}	20.2 ± 1.4 ^{b}	0.65 ± 0.02 ^{b}	68.6 ± 4.0 ^{b}	4.02 ± 0.08 ^{b}

Larval parameters

Log Rank (Mantel-Cox) test showed an overall significantly lower survival of larvae fed with stressed trees in comparison with control ones (Chi^2 = 71.837, df = 1, P < 0.001). Still, pairwise comparisons (i.e., stress vs. control groups) for each PPM population evidenced significant differences only for WP, with survival of the larvae fed on stressed trees decreasing sharply as from the first days of feeding (Fig. 3). By the end of the trial, mortality reached almost 100% for larvae fed on stressed trees, but was also very high for the control ones. Kaplan-Meyer estimates for the average number of days of survival for SP larvae were 21.1 ± 0.7 days and 19.6 ± 0.6 days, respectively for larvae fed on control and stressed trees. However, estimated survival (expressed as the number of days) was lower for the WP larvae than for the SP, especially for those fed on needles from stressed host trees (18.8 ± 0.7 days and 11.5 ± 0.5 days, on control and stressed hosts, respectively).

The mean development time needed to reach the 2nd instar was shorter for larvae fed on control trees (12.3 days ± 0.5) than for larvae fed on stressed trees (13.8 days ± 0.6). However, differences were not significant (Wald Chi^2 = 3.685, P = 0.055). Similarly, differences between PPM population feeding trials were borderline (Wald Chi^2 = 3.630, P = 0.057), with larval development being on average faster in SP than WP. The interaction term was not significant (Wald Chi^2 = 0.193, P = 0.660).

Tree stress had an overall significant negative effect on the needles consumption (Wald Chi^2 = 4.439, *P* = 0.035). Overall, average consumption was 5.48 ± 0.54 mm per larvae.day^{-1} in the control group and 4.00 ± 0.45 mm on the stressed plants (Fig. 4). On average, there were no differences between the SP and WP trials (Wald Chi^2 = 0.121, *P*= 0.728) and the interaction term plant treatment x trial was not significant (Wald Chi^2 = 1.328, *P* = 0.249). Yet, pairwise comparisons between control group and stressed plants showed that differences were higher for WP (*P* = 0.072) than for SP (*P* = 0.256). Concerning the production of feces, no significant effect was found for treatment (Wald Chi^2 = 1.731, *P* = 0.188), population (Wald Chi^2 = 0.003, *P* = 0.957) or the interaction term (Wald Chi^2 = 2.326, *P* = 0.127).

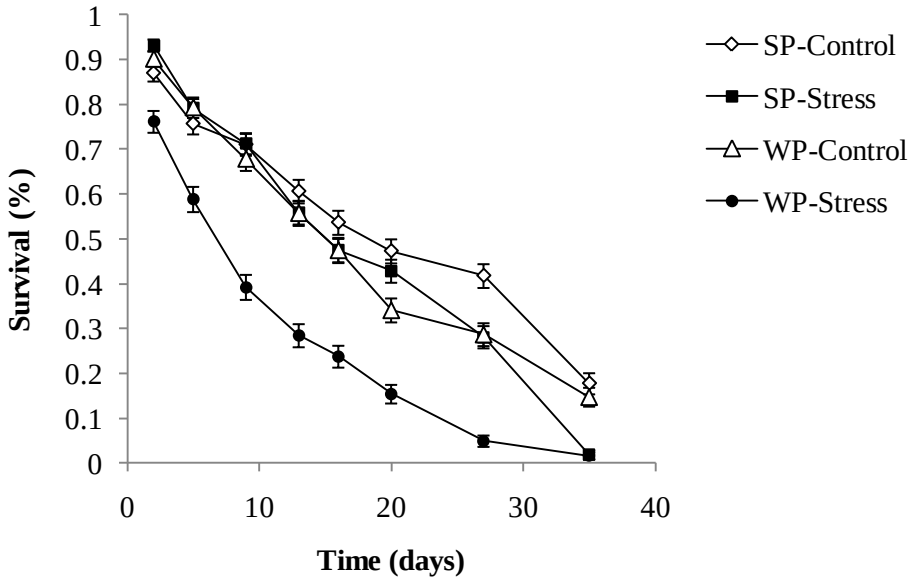

Figure 3 - Larvae survival estimates (± SE) of the two phenotypic divergent populations of *T. pityocampa* (SP and WP) reared with pine needles of drought stress and control trees.

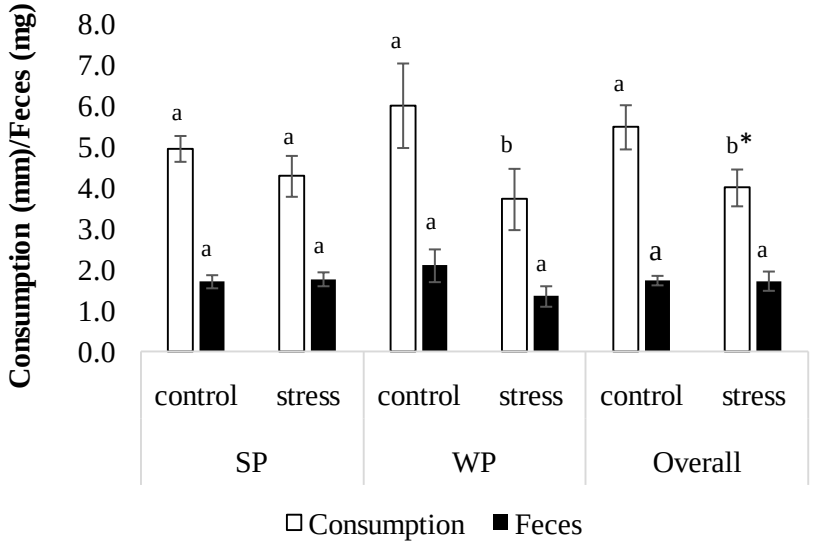

Figure 4 - Consumption (in millimeters) and production of feces (in milligrams) by the larvae of both trials (SP and WP) on drought stressed and control trees (mean ± SE). Different letters mean statistically significant differences between treatments determined by multiple pairwise comparisons through Least Significant Difference (α=0.1 and α=0.05 - indicated with *).

Discussion

Overall, we observed a tendency for a negative effect of plant water stress on larval performance, reflected on a lower larval survival, slower development and lower consumption rate. Yet, a negative effect was significant only for the WP, mainly resulting in a lower larval survival. SP larvae survived, on average, 2 and 8 more days than WP larvae when fed on control and stressed plants, respectively. Taking into account the shifted phenology and the recent and local origin of the SP (Leblois et al. 2017), it can be considered that SP larvae have been adapting to summer dryer conditions.

The negative effect on the performance of the larvae can be explained by changes in plant chemical and physical traits. Water stressed plants registered higher amounts of soluble sugars, higher C/N ratio and phenols; needles were tougher, whereas water content and chlorophylls concentrations were lower. Water stressed trees reached similar stress values and chemical traits for SP and WP trials, which suggest that the differences found in insect performance are not due to a difference in plant treatment.

The leaf water potential of the stressed trees in both trials was in the range of values measured in *P. pinaster* trees under field conditions during the summer (e.g. Loustau et al., 1996).

Most of the results obtained in this work are supported by literature references. Several studies document effects of drought stress in plants reflected in leaf water potential, relative water content, turgor, transpiration rate and photosynthesis (Huberty & Denno, 2004; Jaleel et al., 2009). Consequently, many components of plant nutritional quality are modified with possible effects, either positive or negative, on the performance of associated insects. The outcome may also depend on how insects feed on plant tissues (Huberty & Denno, 2004). Several studies concluded that drought often increases the concentration of sugars and inorganic ions, as well as nitrogen compounds, such as amino acids, in pant tissues thus enhancing foliage nutritional quality for some herbivore insects (Huberty & Denno, 2004; Rouault et al., 2006; Netherer & Schopf, 2010; Jactel et al., 2012). Yet, in the system here studied, the possible positive effect of increased sugar concentration was insufficient to counteract the negative effects of plant drought stress on larvae. At the same time, our results confirmed that a moderate water stress applied to *P. pinaster* trees, led to a significant decrease in chlorophyll concentration and higher C/N ratio, similarly observed by Chen et al. (2015) for sorghum seedlings.

On the other hand, a negative effect on insect performance can occur due to a decrease in leaf water content, increased leaf toughness or to an increase of secondary metabolites concentration. According to Henriksson et al. (2003), leaf water content is one of the most important factors influencing larval growth of *Epirrita autumnata* (Lepidoptera). In our study, stressed plants revealed lower values of leaf water potential and relative water content (RWC) than control ones which could explain in part, the negative effects observed on larval performance. A compensation mechanism was not observed for PPM, as consumption was lower when feeding upon needles of stressed plants than from non-stressed ones. We also found that stressed needles were tougher than control ones, which could explain the lower larval consumption observed in water stressed plants. Interestingly, a previous study (Santos et al., 2013) showed that SP eggs are generally bigger that WP eggs, suggesting that SP neonate larvae are also bigger, with stronger mandibles. This trait could allow SP young larvae to feed more efficiently on tougher needles, and thus perform better than WP larvae on water stressed pines. This could explain the significant difference in survival observed, since the first days

when neonate larvae were present (Fig. 3). A similar hypothesis was suggested by Zovi et al. (2008) who documented a case of local adaptation of *T. pityocampa* larvae to soft vs. tough needles. It is worth noting that overall mortality was very high by the end of the feeding trial, even in the control groups, which is explained by the high sensitivity of this insect species to laboratory rearing conditions during prolonged periods (Berardi et al., 2015; Branco et al., 2017). The production of feces was not affected by plant treatments. This particular outcome, lower consumption but unchanged feces production, suggests a reduction in digestibility of the pine needles following water stress. We could not calculate digestibility indices to confirm this hypothesis due to the gregarious behavior of this insect.

Concentrations of several classes of defensive secondary compounds such as cyanogenic glycosides, phenols, terpenoids, and alkaloids may also increase in plant tissues in response to moderate drought stress (Mattson & Haack, 1987; Kolb et al., 2016). In both trials total phenols were significantly higher in water stressed plants compared to control ones which might have further accounted for the negative effect on larvae, especially on their survival. A higher detoxifying capacity of SP vs. WP larvae could have occurred thus explaining that the negative effect of stress was only significant for the WP. This hypothesis could be explored via dedicated experiments and analyses of differential expression of detoxification genes in both populations in stress vs. control groups.

The overall better performance of SP larvae on stressed plants by comparison to WP, may indicate an evolutionary adaptation to cope with the changes resulting from host plant drought stress. In fact SP larvae feed during summer months, facing environmental conditions such as high temperatures, low air humidity and low rainfall (Fig. 1), all contributing to plant drought stress, whereas WP larvae feed during fall and winter, when the tree's physiological parameters are supposed to be less stressed. Previous studies demonstrated that the SP has already undergone some ecological divergences in relation to the WP, namely regarding the upper thermal tolerance limits of the larvae (Santos et al., 2011b) and of the eggs (Rocha et al., 2017), as well as some reproductive traits, such as fecundity and egg size (Santos et al., 2013). Also, SP larvae had a faster development than WP, with more larvae reaching the 3rd instar in both treatments. Overall, results suggest that the SP is better adapted to develop on stressed hosts and might have a competitive advantage over the WP.

Conclusions

Under present climate change scenarios, characterized by rising temperatures and moderate to severe drought stress episodes, plant drought stress will accordingly increase and modulate plant-insect interactions. This work proved that water stress affected negatively the performance of young larvae of the pine defoliator species studied. A major finding revealed that the SP population, with summer larval feeding, was less affected by stressed trees than the WP population, indicating that some adaptation to the physiological status of the host plant has occurred following the phenological shift. This could be due to stronger mandibles, ability to detoxify secondary compounds and/or capacity to withstand feeding upon low water contents foliage. The present study also highlights the relevance to understand evolutionary and adaptation mechanisms that might occur in the context of global climate change and how this will affect species predictions under different scenarios.

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CONCLUSIONS

Conclusions

This study provided relevant knowledge on the evolutionary and ecological drivers of an allochronic differentiation process experienced by a unique population of the pine processionary moth, *Thaumetopoea pityocampa* (Den. & Schiff.) (Lepidoptera: Notodontidae). The genetic, ecological and geographical characteristics are exceptionally dynamic. Here, we particularly focused on the factors constraining geographical distribution and expansion patterns, on the fine scale spatio-temporal genetic structure and on specific ecological adaptations of this population. Various approaches were used to reach the objectives of the thesis, namely field surveys, experimental approaches, modelling, population genetics, ecology and plant physiology.

The main results can be summarized and synthetized as follows:

Dispersal patterns of the shifted population of T. pityocampa *under current and future climatic conditions*

In this chapter, we explored the potential intraspecific climatic niche divergence between the allochronic WP and SP and made predictions about the evolution of the SP distribution range over time. Following an intensive systematic survey in the field to record the presence/absence of SP nests in the MNL and surrounding places, where the two sympatric populations co-exist, we documented the current distribution of SP. Multivariate analyses and calibrated species distribution models (SDMs) allowed predicting future distribution under different climate change scenarios. The following conclusions can be drawn from our results:

- Spread models estimated a spread rate of SP southward and northward, respectively of 2.51 and 1.66 km year^{-1} over the period 1997–2014. It is anticipated that expansion will continue at similar rate over the following years. In such a scenario, the SP would reach the Lisbon city urban area by 2030. Yet, landscape features as well as rural areas with agricultural land uses and lower continuity of pine forests may either slow the spread or, inversely, accelerate it if the moth takes advantage of isolated and scattered host pines. In fact, in the North of France the expansion of local pine processionary moth was found to be favored by isolated pines, which helps stepping-stone geographical expansion.

- The maximal temperatures of the summer months were identified as the main factor explaining the absence of the SP from inland regions. This climatic variable is also the main constraint acting on the geographic range of this population.
- SDMs globally predict that the current climatic conditions encountered in most lowland regions of the Mediterranean basin would be practically unsuitable for SP establishment. Thus, distribution of SP in Portugal will be limited to a narrow strip along the coast. Its establishment in other countries would thus be highly unlikely as a suitable climatic envelope was only found in a few spots in southern Morocco, Cantabria and the Bask Country.
- Under climate change scenarios considering global warming, all the models predict a future dramatic decrease of the SP range.
- In conclusion, our study clearly demonstrates that the distribution of SP is strongly constrained by environmental parameters and only a small subset of the geographic range of the classical WP (i.e. the Mediterranean basin) is currently climatically suitable for the long term establishment of the SP.

Analysis of the genetic structure of the allochronic SP and WP

The fine-scale temporal and spatial genetic structure of the two allochronic populations, SP and WP, was studied over the whole SP distribution range using a systematic sampling along two transects (one along the coast and one in the inner area). In the same time, a large-scale population study was conducted to decipher the patterns of differentiation over Portugal. This study raised the following conclusions:

- We evidenced a strong pattern of population structure of the pine processionary moth over Portugal, with a significant pattern of isolation by distance. SP is highly differentiated from all other sampled populations.
- No within-population differentiation was found along the two transects across two years, suggesting a high stability over time and space. Similar results were found for the SP and the WP.
- A limited number of SP males emerging each year at the beginning or the end of the WP season were found. 99% of the SP males emerged before mid July each year, but the curve of emergence dates showed a long flat tail. However very few introgressed individuals were detected, suggesting that gene flow is highly

- reduced but not null. Reproductive isolation is thus incomplete between the two populations, as suggested by previous studies.
- The spatial distribution of the two populations showed contrasting patterns. The SP was mostly limited to the costal transect where the WP is now scarce or absent, while the majority of the males trapped over the inner transect belonged to the WP, which suggests competitive exclusion. Under such a scenario, the allochronic differentiation process that probably started in sympatry would be moving toward a parapatric evolutionary process.

Ecological adaptations between the two allochronic populations, regarding embryonic development

This chapter tested the effect of a wide range of maximum temperatures (36 to 48ºC) on egg survival and development of the two sympatric SP and WP Portuguese populations and a Tunisian one. This experimental approach allowed setting the lethal egg temperature for *T. pityocampa* in the case of short-term exposures. We mainly concluded that:

- Simulated heat waves had a severe negative effect on egg survival as temperatures increased. SP was the least affected population as more embryos survived the heat treatments, suggesting an adaptive process in this population and a higher success at the egg stage.
- Egg development time was also affected by increasing temperatures, and was longer for higher temperatures.
- The maximal temperature threshold of survival was determined to be 42ºC, as no embryo survived for any of the tested populations above this limit. The lethal maximal temperature for the *T. pityocampa* eggs was so far unknown.. This information is most important to incorporate in SDMs and to further tune the prediction of future distributions of the pine processionary moth under climate change scenarios.

Potential adaptive response of the allochronic populations, regarding host plant hydric status

This chapter studied how larvae of the SP and the WP cope with host plant stress, and if phenotypic differences could be identified regarding this constraint:

- Larvae of both populations had lower performances on water stressed plants, as shown by lower survival rate, lower needle consumption and longer development times.
- The detrimental effects of tree water stress on the larvae were significant only for WP, while SP was proved to cope better with host drought stress conditions.
- The study evidenced intra-specific variations on the response of this insect to host plant status and adaptive mechanisms at population level.

Twenty years after the first discovery of this atypical population in MNL in 1997, our knowledge about the origin, evolution and ecological adaptation of this unique population has greatly increased. The work presented in this thesis brought new light on the future fate of this population and on the speciation process and phenotypic differentiation. We highlighted the complexity of the speciation process, and provided a fine description of spatial and temporal neutral genetic differentiation, as well as evidence of adaptive evolution. We also identified the climatic parameter constraining the SP spatial range. Still, some questions regarding SP remain open, requiring future research. In particular the hypothesis of competitive exclusion of the two phenological types (SP/WP) suggested in the present study needs to be investigated, and the distribution of nests have to be recorded to confirm the hypothesis of recent evolution toward parapatry. The role of the natural enemies and their possible asynchrony with SP, which could now develop in an enemy-free space, was only scarcely addressed in the egg stage and deserves further research. The exact scenario about the origin and demographic history of the SP is still unknown, and will be addressed in a future work (Leblois et al., in preparation). Also, the genetic mechanisms involved in the shift of the life cycle are still unknown. Candidate genes involved in the onset or termination of diapause should now be explored, and future genomic and transcriptomic studies will be developed. To this end, a reference genome and transcriptome have been assembled. They will be useful to identify the underlying genomic mechanisms, but will require

specific competences. The consequences of a summer defoliation for the trees were not addressed so far. Societal and economic consequences of a population with such a particular life cycle have not been considered, although we anticipate that the summer activity of the larvae will bring higher concern to human activities, in particular due to the presence of urticating larvae in summer. This concern will be higher in the future, as we predicted that the SP would reach the urbanized area of Lisbon in the next decade. We expect that further studies will help to clarify some of these questions.